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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
07039-167002**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)

10/030585INTERNATIONAL APPLICATION NO.
PCT/US00/40324INTERNATIONAL FILING DATE
7 July 2000PRIORITY DATE CLAIMED
9 July 1999

TITLE OF INVENTION

CFTR POLYPEPTIDES, FRAGMENTS THEREOF AND METHODS OF USE TO OVERCOME BIOSYNTHETIC MISPROCESSING

APPLICANT(S) FOR DO/EO/US

John R. Riordan and Xiu-bao Chang

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other documents or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - ☒ Return Postcard
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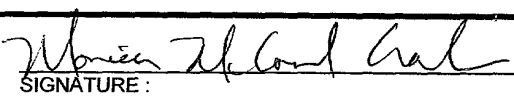
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8 January 2002
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Typed Name of
Person Signing

U.S. APPLICATION NO. (IF KNOWN) 10/030585		INTERNATIONAL APPLICATION NO. PCT/US00/40324		ATTORNEY'S DOCKET NUMBER 07039-167002	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$710.00	
Surcharge of \$130 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	53 - 20 =	33	x \$18	\$594.00	
Independent Claims	14 - 3 =	11	x \$84	\$924.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ \$280	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$2,228.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$1,114.00	
SUBTOTAL =				\$1,114.00	
Processing fee of \$130 for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$0.00	
TOTAL NATIONAL FEE =				\$1,114.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$0.00	
TOTAL FEES ENCLOSED =				\$1,114.00	
				Amount to be refunded:	\$
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a. <input checked="" type="checkbox"/> A check in the amount of \$1,114.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 06-1050 in the amount of \$0.00 to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Mark S. Ellinger, Ph.D. FISH & RICHARDSON P.C., P.A. 60 South Sixth Street Suite 3300 Minneapolis, MN 55402 (612) 335-5070 phone (612) 288-9696 facsimile			<div style="text-align: center;">  SIGNATURE: </div> <div style="text-align: center;"> NAME Monica McCormick Graham, Ph.D. </div> <div style="text-align: center;"> REGISTRATION NUMBER 42,600 </div>		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John R. Riordan, et al. Art Unit : Unknown
Serial No. : Not Yet Assigned Examiner : Unknown
Filed : Herewith
Title : CFTR POLYPEPTIDES, FRAGMENTS THEREOF AND METHODS OF USE
TO OVERCOME BIOSYNTHETIC MISPROCESSING

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the claims:

Cancel claims 4 and 44 (second occurrence).

Please amend claim 7:

7. (Amended) A polynucleotide encoding the polypeptide of claim 3.

Add claims 50-53.

- 50. The method of claim 43, wherein said protein is secreted.
51. A polynucleotide encoding the polypeptide of claim 1.
52. A polynucleotide encoding the polypeptide of claim 2.
53. A pharmaceutical formulation comprising an isolated polypeptide selected from the group consisting of GYRQRLE, EYRYRSV, GQRARIS, and QARRRQS.--

CERTIFICATE OF MAILING BY EXPRESS MAIL

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Vince Defante
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Applicant : John R. Riordan, et al.
Serial No. : Not Yet Assigned
Filed : Herewith
Page : 2

Attorney's Docket No.: 07039-167002

REMARKS

The application was amended to correct a typographical error and to remove multiple dependent claims.

Attached is a marked-up version of the changes being made by the current amendment.

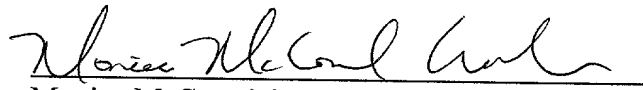
Applicant asks that all claims be examined. Enclosed is a check for excess claim fees.

Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date:

1/8/02



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60067628.doc

Version with markings to show changes made

In the claims:

Claims 4 and 44 (second occurrence) have been cancelled.

Claim 7 has been amended as follows:

7. (Amended) A polynucleotide encoding the polypeptide of claim[s 1, 2
or] 3.

10/030585

**CFTR POLYPEPTIDES, FRAGMENTS THEREOF AND METHODS OF USE
TO OVERCOME BIOSYNTHETIC MISPROCESSING**

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with Government support awarded by the National
5 Institutes of Health. The Government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

The invention relates to biosynthetic maturation of cell surface polypeptides
and, more specifically, to particular CFTR polypeptides which exhibit increased
transport to the cell surface and tripeptide amino acid sequences that promote or
10 enhance transport of export-incompetent CFTR to the cell surface.

Large multidomain and multisubunit proteins are assembled into their native
tertiary and quaternary structures, respectively, in the endoplasmic reticulum (ER). If
this assembly is imperfect because of mutations or for other reasons, the aberrant
protein is targeted for degradation by a set of processes generally referred to as
15 biosynthetic quality control. Although a great deal has been learned about these
processes in the past few years the rate-limiting step determining whether or not a
nascent chain is exported from the ER has not been identified. Indeed, the relation of
the folding and degradation mechanisms to the ER export and retrieval pathways is
not understood for any cell surface or secreted molecule.

20 Cystic fibrosis (CF) is an example of a disease which may benefit from an
understanding of the factors that contribute to or mediate retention or export of
proteins from the endoplasmic reticulum because, in many patients, ER-retention
prevents potentially functional variant CFTR (Cystic Fibrosis Transmembrane
Conductance Regulator) molecules from reaching the plasma membranes of secreting
25 and reabsorbing epithelial cells, where CFTR is required as a regulated chloride
channel. For example, although more than 800 different mutations in the CFTR gene
have been detected in CF patients, more than 90% of patients possess a single
misprocessing mutant, $\Delta F508$. Collins, F. S. (1992) *Science* 256:774-779. This
mutant protein is potentially functional as a regulated chloride channel if it can be
30 made to move further along the secretory pathway and reach the cell surface.

Dalemans, W. et al. (1991) Nature 354:526-528; Drumm, M. L. et al. (1991). Science 254:1797-1799; and Li, C. et al. (1993) Nat. Genetics 3:311-316.

Ubiquitination ultimately marks maturation-incompetent nascent chains as substrates for the proteasome but earlier steps in the recognition of these targets are unclear. Jensen, T. J. et al. (1995) Cell 83:129-135; Sato, S. et al. (1998). J. Biol. Chem. 273:7189-92; and Ward, C. L et al. (1995) Cell 83:121-127. Molecular chaperones can either retain unfolded proteins or assist in their folding. Nascent CFTR interacts with chaperones on both sides of the ER membrane; on the cytoplasmic face of the ER, Hsp70 (Yang, Y. et al. (1993) Proc. Natl. Acad. Sci. USA 90:9480-9484) and its cochaperone, Hdj-2 (Meacham, G. C. et al. (1999) EMBO J. 18:1492-1505) as well as Hsp90 (Loo et al. (1998) EMBO J. 17:6879-6887) bind to immature CFTR. Other chaperones could also be present in the large multimolecular complexes containing nascent CFTR. Pind, S. et al. (1994) J. Biol. Chem. 269:12784-12788. Although these interactions appear to occur with both wild-type CFTR and mutant $\Delta F508$ CFTR and no large differences in the kinetics or stoichiometry of CFTR-chaperone interactions have yet been found, the role of chaperones in ER retention of CFTR cannot be ruled out.

In addition to chaperones, short sequence motifs have been shown to exert positive and negative effects on secretory proteins which are required for ER export and retrieval, respectively. For example, a short diacidic ER export signal has been described as necessary for transport of VSV-G glycoprotein from the ER. Nishimura, N., and Balch, W. E. (1997) Science 277:556-8. Whether nascent $\Delta F508$ CFTR ever leaves the ER or if it is retrieved is not known, although $\Delta F508$ CFTR may reach the intermediate compartment (ERGIC) between ER and Golgi. Gilbert, A. et al. (1998) Exp. Cell Res. 242:144-52.

Previous attempts to overcome ER-retention of mutant CFTR have included inhibition of the proteasome involved in nascent chain proteolysis (Jensen et al., 1995, supra; Ward et al., 1995, supra), perturbation of interaction with molecular chaperons (Jiang, C. et al. (1998). Am. J. Physiol. 275:C171-8; and Loo et al., 1998, supra) and using agents or conditions which influence protein folding for example, glycerol and other osmolytes (Brown, C. R. et al. (1996) Cell Stress Chaperones 1:117-25; Qu, B. H. et al. (1997) J. Biol. Chem. 272:15739-44; and Sato, S. et al. (1996) J. Biol. Chem. 271:635-638) or reduced temperature (Denning, G. M. et al. (1992) Nature 358:761-

764). However, these treatments are minimally effective or extremely toxic to cells, precluding their application to patients.

Thus, there remains a need to understand the biological basis for ER retention, especially in respect to the retention of proteins such as CFTR that have severe physiological consequences. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that an export-incompetent CFTR has several arginine-framed tripeptide sequence motifs that contribute to the observed deficiency of cell surface expression, and that modifying one or more of these sequences can result in increased transport of the export-incompetent CFTR to the cell surface.

In one aspect, the invention features an isolated polypeptide that includes a polypeptide having the sequence X1-R-X2-R-X3, wherein X1, X2 and X3 are any amino acid and polynucleotides encoding such polypeptides. The polypeptide can have at least seven amino acids. The invention also features an isolated polypeptide selected from the group consisting of: GYRQRLE, EYRYSV, GQRARIS and QARRRQS, and polynucleotides encoding such polypeptides. Polypeptides of the invention further can include a pharmaceutical formulation.

The invention also features an isolated polypeptide having one or more R-X-R sequences, wherein at least one R of an R-X-R sequence has been substituted with another amino acid, and wherein the substituted polypeptide is exported from the ER in an amount or at a rate greater than the unsubstituted polypeptide. The polypeptide can be CFTR.

In another aspect, the invention features a method for identifying a therapeutic agent for treating cystic fibrosis. The method includes contacting at least one cell expressing an export-incompetent CFTR with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence; and determining whether the agent increases the amount of the CFTR on the cell surface, where an increased amount of CFTR on the cell surface identifies a therapeutic agent for treating cystic fibrosis.

In yet another aspect, the invention features a method for identifying an agent that induces or increases transport of an export-incompetent protein. The method includes contacting an export-incompetent protein with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence; and determining whether the agent increases the amount or the rate of protein transported, where an increased amount of transported protein identifies an agent that induces or increases transport of an export-incompetent protein. The protein can be a secreted protein, a cell surface protein, or CFTR. The method can be performed in vitro or the export-incompetent protein can be expressed in a cell.

The invention also features a method for treating a subject having cystic fibrosis. The method includes administering to the subject a pharmaceutical formulation comprising a polypeptide having an R-X-R sequence in an amount effective for treating cystic fibrosis. In one embodiment, the X amino acid of the R-X-R sequence is not alanine, asparagine or glutamate. The polypeptide can have at least four amino acids, e.g., at least seven amino acids. The polypeptide can be selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

In yet another aspect, the invention features a method for treating a subject having cystic fibrosis. The method includes administering to the subject a pharmaceutical formulation that includes a nucleic acid encoding a polypeptide having an R-X-R sequence in an amount effective for treating cystic fibrosis.

In another aspect, the invention features a method for treating a subject having or suspected of having a physiological disorder associated with an export-incompetent protein. The method includes administering to the subject a pharmaceutical formulation comprising a polypeptide having an R-X-R sequence in an amount effective for treating a physiological disorder associated with an export-incompetent protein. The physiological disorder or condition can be selected from the group consisting of: macular dystrophy and Stargardt's disease. The export-incompetent protein can be selected from the group consisting of: ion channels, ABC proteins, growth factors, immune regulators, adhesion proteins, hormones, clotting factors, hemostatic regulators and receptors thereof. In one embodiment, the X amino acid of

the R-X-R sequence is not alanine, asparagine or glutamate. The polypeptide can have at least four amino acids, e.g., at least seven amino acids. The polypeptide can be selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

5 In yet another aspect, the invention features a method for inducing or increasing intracellular transport of an export-incompetent protein. The method includes contacting a cell expressing an export-incompetent protein with a composition comprising a polypeptide having an R-X-R sequence in an amount sufficient for inducing or enhancing intracellular transport of the export-incompetent protein. The protein can have an R-X-R sequence and can be a cell surface protein, an export-incompetent CFTR, or a secreted protein.

10 A method for identifying an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER retention factor also is described. The method includes incubating a polypeptide having an R-X-R sequence and an ER retention factor under conditions allowing their interaction; adding a test agent to the incubation; and detecting binding between the polypeptide and the ER retention factor, where decreased binding in the presence of the test agent identifies an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER retention factor. The method can be performed in vitro. The polypeptide can have at least 4 amino acids. The X amino acid of the R-X-R sequence may not be alanine, asparagine or glutamate. The polypeptide can be selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

15 The invention also features a method for identifying an ER retention factor. The method includes contacting a composition suspected of containing an ER retention factor with a polypeptide having an R-X-R sequence under conditions allowing interaction between the factor and the polypeptide; and detecting binding between the factor and the polypeptide, thereby identifying an ER retention factor. The method can be performed in vitro. The polypeptide can have at least four amino acids, e.g., at least seven amino acids. The polypeptide can be selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

20 In yet another aspect, the invention features a method for inhibiting degradation of a protein in a cell. The method includes contacting a cell with a polypeptide having an R-X-R sequence in an amount sufficient for inhibiting

degradation of a cell surface or secreted protein. The protein can be a cell surface protein or a secreted protein.

A method for detecting the presence of an export-incompetent protein in a cell is also featured. The method includes contacting a cell with a polypeptide having an R-X-R sequence and detecting the intracellular transport of the protein. The intracellular transport can be detected using an enzyme, by detecting the presence of the protein on the cell surface, or by detecting secretion of the protein. The protein can be CFTR.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1D show A) a schematic depiction of CFTR protein indicating approximate locations of arginine-framed tripeptide sequences and phenylalanine 508; B) western blots of wild-type and $\Delta F508$ CFTR expressed in BHK cells having the indicated substitutions; C) wild-type and $\Delta F508$ CFTR expressed in BHK cells having all four arginines substituted with lysine ($\Delta F508/4RK$) digested with endoglycosidase H; and D) surface biotinylation of mature CFTR on cells expressing $\Delta F508$, $\Delta F508/4RK$ and wild-type CFTR. The arrowhead indicates position of mature band with complex (endoH resistant) oligosaccharide chains. Solid arrows indicate the position of core-glycosylated band before endo-H digestion and open arrows are after endo-H digestion. Amino acid numbering begins from the first methionine in CFTR. Expression, extraction, digestion and cell surface labeling was performed as described in Example I.

Figures 2A to 2D show the maturation of A) wild-type CFTR; B) wild-type CFTR with arginine to lysine substitutions at all four arginine-framed tripeptides (wild-type/4RK; C) $\Delta F508$ CFTR and; D) $\Delta F508$ CFTR with arginine to lysine substitutions at all four arginine-framed tripeptides ($\Delta F508/4RK$). Cells were pulse-labeled for 20 min and the chase times in hours are indicated. Maturation was assessed by the acquisition of endoglycosidase H resistant oligosaccharide chains, which results in a decrease in the corresponding proteins mobility on SDS-PAGE.

Figures 3A to 3D show the localization of A) wild-type CFTR; B) wild-type CFTR with arginine to lysine substitutions at all four arginine-framed tripeptides; C) $\Delta F508$ CFTR and; D) $\Delta F508$ CFTR with arginine to lysine substitutions at all four arginine-framed tripeptides, as determined by indirect immunofluorescence.

Figures 4A to 4D show the effect of AFT-containing heptamers on cells expressing wild-type CFTR-GFP fusion protein and $\Delta F508$ CFTR-GFP fusion protein. A) cells expressing wild-type CFTR-GFP fusion protein, not exposed to AFT-containing heptamer peptides; B) cells expressing wild-type CFTR-GFP fusion protein, exposed to AFT-containing heptamer peptides; C) cells expressing $\Delta F508$ CFTR-GFP fusion protein, not exposed to AFT-containing heptamer peptides; D) cells expressing $\Delta F508$ CFTR-GFP fusion protein, exposed to AFT-containing heptamer peptides.

Figures 5A and 5B show the effect of the arginine to lysine substitutions on CFTR chloride channel activity from A) cells expressing wild-type and $\Delta F508$ CFTR with or without AFT mutations ($^{36}\text{Cl}^-$ efflux in cpm/min) and B) single CFTR Cl^- channels in planar lipid bilayers isolated from cells expressing wild-type, wild-type/4RK and $\Delta F508$ /4RK CFTR. The studies in A) were performed in triplicate, samples were averaged and the vertical bars represent standard deviation. In B), the arrows indicate stimulation with Forskolin and all points histograms are shown to the left of the tracings.

Figure 6 is an amino acid sequence of human cystic fibrosis transmembrane conductance regulator (CFTR).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the discovery that particular mutant CFTR's are unable to be transported to the cell surface due, in part, to the presence of arginine-framed tripeptide sequence motifs, R-X-R, present in CFTR. There are four arginine-framed tripeptide sequence motifs on CFTR that appear to mediate or contribute to retention of mutant export-incompetent CFTR in the endoplasmic reticulum (ER), delaying or preventing transport of CFTR to the cell surface. When altered, such as by substituting one arginine of the tripeptide sequence motif with another amino acid, an increase in transport of the substituted CFTR to the cell surface in an amount greater than unsubstituted export-incompetent CFTR is observed. Thus, agents that induce or increase transport of export-incompetent CFTR or other export-incompetent proteins from the ER can be used as therapeutics for treating physiological diseases associated with export-incompetent proteins, such as cystic fibrosis (CF).

In one embodiment, the invention provides isolated CFTR polypeptides having one or more amino acid substitutions of an arginine-framed tripeptide (AFT). The substituted CFTR polypeptides are capable of being transported to the cell surface in an amount greater than an unsubstituted CFTR polypeptide. Thus, substituted CFTR polypeptides are useful for the treatment of CF, for example. Moreover, as AFTs can mediate or contribute to a protein's inability to be transported from the ER, AFT and peptide sequences containing such AFT sequences are useful as competitive inhibitors of ER retention factors that interact with AFT containing polypeptides. As disclosed herein, competitive inhibition using such peptides induces or enhances ER transport of proteins, including export-incompetent proteins containing an arginine-framed tripeptide sequence. Thus, arginine-framed tripeptides are useful for treating physiological disorders related to export-incompetent proteins, including cell surface as well as secreted proteins, such as CFTR. Thus, in another embodiment, the invention provides peptides having one or more arginine-framed tripeptide sequences, and methods of use.

As used herein, the terms "protein," "polypeptide" and "peptide" are used interchangeably to denote an amino acid polymer that comprises at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (*e.g.*, glycosylation, phosphorylation, lipidation, ubiquitination etc.). D- and L- amino acids, and mixtures thereof are included.

As used herein, the terms "isolated" or "substantially pure," when used as a modifier of invention CFTR polypeptides, fragments thereof, arginine-framed tripeptides and nucleic acids, means that they are produced by the hand of man and are separated from their native *in vivo* cellular environment. Generally, polypeptides and nucleic acids so separated are substantially free of other proteins, nucleic acids, lipids, carbohydrates or other materials with which they are naturally associated.

Typically, a polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 95%, by weight. Substantially pure CFTR polypeptide can be obtained, for example, by extraction from a natural source (*e.g.*, an animal cell); by expression of a recombinant nucleic acid encoding a CFTR

polypeptide: or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

As disclosed herein, substitution of one or more amino acids of an AFT sequence with a different amino acid can increase transport of an export-incompetent polypeptide (see for example, Examples I to III). Thus, in another embodiment, the invention provides an isolated polypeptide containing at least one arginine-framed tripeptide, R-X-R, in which at least one R of the R-X-R tripeptide is substituted with an amino acid, and the substituted polypeptide is exported from the endoplasmic reticulum in an amount greater than the unsubstituted polypeptide. In one aspect, the first or third R is substituted or the X position amino acid is substituted. In another aspect, the first or third R is substituted with a lysine. The X position amino acid is not substituted with alanine, asparagine or glutamate.

Particular examples of isolated polypeptides so substituted include export incompetent Δ F508 CFTR, for example, R29K and R516K, in which the first arginine has been substituted with a lysine, R555K and R766K, in which the third arginine has been substituted with a lysine, and 4RK, in which all four substitutions are combined (Figure 1B). Additional modifications of the CFTR sequence may influence ER-retention or export. For example, substitution of transport signals within the CFTR C-terminal extension residues (amino acid residues 1380-1480) also can increase ER export of an export-incompetent CFTR.

As used herein the term "substituted," when used as a modifier of a polypeptide containing at least one arginine-framed tripeptide (R-X-R), means that one or more amino acids of the R-X-R sequence or a sequence that flanks R-X-R, has been substituted with another amino acid. Preferred amino acid substitutions occur at the first or third arginines of the R-X-R motif. More preferred substitutions occur at the first arginine of the R-X-R motif. Generally, substitutions of the sequence

flanking R-X-R will be within about 15 amino acids of the motif, preferably within 10 amino acids and more preferably within five amino acids. Multiple amino acid substitutions of R-X-R or a sequence that flanks R-X-R also are included.

As used herein, the terms "export-incompetent" or "export-incompetence," when used as a modifier of a protein, polypeptide or peptide, refers to a decrease or absence of intracellular transport from the endoplasmic reticulum (ER) to and through the golgi apparatus (cis, medial, trans) to an ultimate destination, such as the cell surface, to the lysosomes or endosomes, or secreted from the cell. A decrease either can occur in the rate of transport (e.g., a time delay in the appearance of a protein on the cell surface) or in the overall amount of protein transported to its ultimate destination, or a combination thereof. As disclosed herein, the rate or amount of intracellular transport of a protein can be determined by detecting the amount of protein present at the cell surface or present in organelles distal to the ER, such as the golgi (e.g., cell surface immunoprecipitations or cell immunocytochemistry using antibody specific for the protein, Examples I to III), for secreted proteins, detecting the amount in the media, or detecting protein transport through the various ER to golgi transport stages, for example, by assaying polypeptides for the presence of complex oligosaccharide chains added by glycosyl transferases present in the golgi. For example, as described in Example I and shown in Figures 1 and 2, pulse-chase studies revealed that wild type CFTR maturation begins within an hour (acquires complex oligosaccharide chains resistant to endoglycosidase H digestion, i.e., "endo H resistant chains"), whereas export-incompetent $\Delta F508$ CFTR fails to mature even after two hours (does not acquire endoglycosidase H resistant chains; compare Figure 2A to 2C). Additional methods, both *in vivo* and *in vitro*, are known in the art that can be used for detecting an increase or decrease in intracellular transport of a protein.

The isolated invention polypeptides having a substituted R of the R-X-R will generally be proteins that are naturally present in the intracellular transport apparatus during their maturation and are therefore normally present on the cell surface, in lysosomes or endosomes, or are secreted by the cell. Thus, such polypeptides include cell surface ion channels, growth factors, immune response regulators receptors (cytokines and growth factors including interleukins, interferons etc.), adhesion proteins (e.g., integrins and galectins), hormones, clotting factors, hemostatic effectors

and receptors thereof. Nevertheless, other polypeptides that are not naturally present in the intracellular transport apparatus, but are modified so as to be present in the intracellular transport apparatus, such as by attaching an appropriate targeting signal, also are included, so long as substituted polypeptide, as set forth herein, is exported to the cell surface in an amount greater than the unsubstituted polypeptide.

Polypeptides containing at least one arginine-framed tripeptide (AFT) in which at least one of the arginines has been substituted with another amino acid include export-incompetent polypeptides as well as polypeptides for which no export deficiency is apparent (*e.g.*, wild-type proteins). Both naturally occurring and non-naturally occurring substituted polypeptides are included. Specific examples of polypeptides that can be substituted are $\Delta F508$ CFTR polypeptides in which an arginine residue at position 29, 516, 555, 766 has been substituted with lysine. Specific examples of polypeptides for which no export deficiency is apparent are wild-type CFTR's in which one or more arginines of the arginine-framed tripeptides have been substituted. For example, wild-type CFTR in which four arginines have been substituted with lysine (wild-type/4RK) appears to undergo more rapid maturation than unsubstituted wild-type CFTR (compare Figure 2A to 2B). As with substituted export-incompetent CFTR, wild type CFTR polypeptides can have one or more substitutions of the arginine at the indicated positions, or have multiply substituted arginines, in any combination. Additional export-incompetent CFTR polypeptides are known in the art and are specifically included when substituted as set forth herein.

The invention further includes polypeptides having minor variations, additions or deletions to the amino acid sequence of the polypeptides disclosed herein so long as the modified polypeptide has substantially the same biological activity or function as the unmodified polypeptide. As used herein, the term "substantially the same biological activity or function," when used in reference to a modified polypeptide, means that the polypeptide retains sufficient biological activity associated with the unmodified polypeptide as described herein or known in the art to provide normal tissue function.

Modified polypeptides are therefore distinct from substituted polypeptides because modified polypeptides can have modifications throughout the polypeptide

that do not destroy biological activity, whereas substituted polypeptides have one or more amino acids of the R-X-R sequence or a flanking sequence substituted with another amino acid, where the substituted polypeptide is exported to the cell surface in an amount or at a rate greater than the unsubstituted polypeptide. Thus, a substituted polypeptide as set forth herein can be modified so long as the polypeptide is exported from the ER in an amount or at a rate greater than the unsubstituted polypeptide. For example, a substituted export-incompetent CFTR polypeptide having modifications would be exported from the ER in an amount or at a rate greater than unsubstituted export-incompetent CFTR. Similarly, a substituted wild-type CFTR having modifications would be exported to the cell surface in an amount greater than unsubstituted wild-type CFTR.

Modified polypeptides that are "biologically active" or "functional" can be identified through a functional assay. For example, modified CFTR will exhibit substantial chloride efflux. An example of a functional assay for a polypeptide having an AFT, would be the ability of the modified AFT to induce or increase transport of an export incompetent protein. Additional functional assays for CFTR are known in the art and various assays for determining whether a modified polypeptide, such as a cell surface or secreted protein, has a biological function or activity include ion transport, ligand binding, cell signaling, the ability to bind or interact with proteins *in vitro* or *in vivo*, enzymatic activity, gene activation or suppression and the ability to be modulated by agents or proteins, for example.

As modified polypeptides will retain biological activity associated with unmodified polypeptide, modified polypeptides will generally have an amino acid sequence "substantially identical" to the amino acid sequence of the unmodified polypeptide. As used herein, the term "substantially identical," when used in reference to a polypeptide, means that a sequence of the polypeptide is at least 50% identical to a reference sequence. Modified polypeptides and substantially identical polypeptides will have at least 70%, preferably 88%, more preferably 90%, and most preferably 95% homology to a reference polypeptide. For polypeptides, the length of comparison between sequences will generally be at least 15 amino acids, preferably at least 25 amino acids, more preferably at least 50 amino acids, and most preferably 100 amino acids or more.

As used herein, the terms "homology" or "homologous," when used in reference to polypeptides, refers to the amino acid sequence similarity between two polypeptides. When an amino acid position in both of the polypeptides is occupied by identical amino acids, then they are homologous at that position. Thus, by
5 "substantially homologous" is meant an amino acid sequence that is largely, but not entirely, homologous, and which retains most or all of the activity as the sequence to which it is homologous.

Modified or substantially identical polypeptides can have one or more additions, deletions, or insertions, or non-conservative variations, located at positions
10 of the amino acid sequence which do not destroy the function of the protein (as determined by functional assays, *e.g.*, as described herein) or conservative variations, for example, one amino acid can be substituted for another of the same class (*e.g.*, valine for glycine, arginine for lysine, etc.).

An example of an addition is a heterologous domain that imparts a distinct
15 functionality upon the polypeptide. A heterologous domain can be any small molecule, macromolecule or microfabricated device so long as it imparts an additional function. Particular heterologous domains include those that provide a targeting function (*e.g.*, an antibody, ligand, viral envelope protein), those that enhance or suppress activity (a derepressible or activatable moiety), and those that enable
20 purification (*e.g.*, T7 tag, polyhistidine sequence etc.). The skilled artisan will know of other heterologous domains depending on the application and the distinct function desired.

An example of a deletion is where a small portion of the molecule is removed. For example, deletion of an AFT may not alter CFTR biological activity, but may
25 induce or increase transport from the ER. Multiple deletions of AFTs may provide an additive or synergistic effect. Polypeptides having AFT deletions are specifically included so long as the deleted polypeptide has increased transport in comparison to undeleted polypeptide.

As used herein, the term "conservative variation" denotes the replacement of
30 an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar

residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

Modified polypeptides further include "chemical derivatives," in which one or more of the amino acids therein has a side chain chemically altered or derivatized.

5 Such derivatized polypeptides include, for example, amino acids in which free amino groups form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups; the free carboxy groups form salts, methyl and ethyl esters; free hydroxyl groups that form O-acyl or O-alkyl derivatives as well as naturally occurring amino acid derivatives, for example, 4-hydroxyproline for proline, 5-hydroxylysine for lysine, 10 homoserine for serine, ornithine for lysine etc. Also included are D-amino acids and amino acid derivatives that can alter covalent bonding, for example, the disulfide linkage that forms between two cysteine residues that produces a cyclized polypeptide.

The polypeptide modifications may be deliberate, as by site-directed (*e.g.*, PCR based) or random mutagenesis (*e.g.*, EMS) or may be spontaneous or naturally 15 occurring. For example, naturally occurring allelic or splice variants can arise by alternative RNA splicing, polymorphisms or spontaneous mutations of a nucleic acid. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant polypeptide without significantly altering a biological activity. Deletion can lead to the development of a smaller active molecule that may 20 have broader utility. Those of skill in the art will recognize the numerous amino acids that can be modified or substituted with other chemically similar residues without substantially altering biological activity. All of the polypeptides produced by such modifications are included herein as long as the modified polypeptide possesses most or all of a biological activity or function as described herein.

25 As the AFT's of CFTR are disclosed herein as contributing to ER retention or delay in maturation of proteins, the AFT sequences are useful as competitive inhibitors of ER retention. Thus, in another embodiment, the present invention provides isolated polypeptides having an AFT sequence. The amino acid length of polypeptides that contain the AFT can range from about 4 amino acids up to about 50 30 amino acids. Preferably, the amino acid length is from about 5 to about 40 amino acids, more preferably, from about 6 to about 30 amino acids and, most preferably, from about 7 to about 25 amino acids. For example, the polypeptide can have the

sequence X_1 -R- X_2 -R- X_3 where X_1 , X_2 and X_3 are any amino acid. In certain aspects, the X_2 amino acid is not alanine, asparagine or glutamate.

Exemplary polypeptides having an AFT sequence are heptamers based on the AFT sequence region of the corresponding CFTR polypeptide. In particular,
5 GYRQRLE, which is based on the sequence SWTRPILRKGYRQRLELSDIYQIPS;
EYRYRSV, which is based on the sequence NIIFGVSYDEYRYRSVIKACQLEED;
GQRARIS, which is based on the sequence GEGGITLSGGQRARISLARAVYKDA;
and QARRRQS, which is based on the sequence
SVISTGPTLQARRRQSVLNLMTHSV. It is understood that the standard single
10 letter amino acid abbreviation is used to denote the amino acids of the invention
polypeptides (see for example, Zubay, G.L., *Biochemistry* page 12, Addison-Wesely
Publishing, Inc., 1983).

AFT containing polypeptides can have multiple AFT sequences, if desired.
AFT polypeptides of the invention also can be modified as set forth herein so long as
15 a biological activity or function is substantially the same as unmodified polypeptide.
Additional modifications further include, for example, those that enhance or increase
activity, so as to be more effective inhibitors of ER retention.

The polypeptides of the invention can be prepared by a variety of methods
known in the art, such as by purification from an appropriate organism or cell using
20 typical biochemical methods (e.g., column chromatography), by chemical synthesis
(peptide synthesizers, e.g. Applied Biosystems, Inc., Model 540A (Foster City, CA)),
by expression screening (using an antibody that binds to the polypeptide). An
example of one means is by expression of a nucleic acid in a cell encoding an
invention polypeptide in a host cell, such as bacteria, yeast or mammalian cell, and
25 purifying the expressed polypeptide using methods known in the art. Other well
known methods are described in Deutscher et al., (Guide to Protein Purification:
Methods in Enzymology Vol. 182, Academic Press (1990)).

The invention further provides isolated nucleic acids encoding invention
polypeptides, fragments thereof, complementary sequences thereto, and antisense
30 sequences thereof. In one embodiment, invention isolated nucleic acids encode export
incompetent CFTR's in which an R of the R-X-R motif is substituted, for example,
with lysine. In another embodiment, invention isolated nucleic acids encode wild-
type CFTR's in which an R of the R-X-R motif is substituted, for example, with

lysine. In certain other aspects, nucleic acids encoding polypeptides having an AFT sequence also are provided.

As used herein, the terms "nucleic acid," "polynucleotide," "oligonucleotide," or "primer" are used interchangeably to refer to deoxyribonucleic acid (DNA) or ribonucleic (RNA), either double or single stranded, linear or circular. RNA can be unspliced or spliced mRNA, rRNA, tRNA or antisense RNA. DNA can be complementary DNA (cDNA), genomic DNA, or an antisense. Specially included are nucleotide analogues and derivatives, such as those used to provide resistance to degradation by nucleases, which can function as antisense or encode invention polypeptides.

An "isolated" or "substantially pure" nucleic acid means that the nucleic acid is not immediately contiguous with the coding sequences with either the 5' end or the 3' end with which it is immediately contiguous in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA that is a separate molecule independent of other sequences (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) as well as a recombinant DNA incorporated into a vector, an autonomously replicating plasmid or virus; or a genomic DNA of a prokaryote or eukaryote. It also includes a recombinant DNA that is part of a hybrid or fusion, for example, a gene encoding an additional polypeptide sequence. The term therefore does not include nucleic acids present among millions of sequences in a genomic or cDNA library, or in a restriction digest of a library fractionated on a gel.

The nucleic acids of the invention also include nucleic acids that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Degenerate sequences may not selectively hybridize to other invention nucleic acids; however, they are nonetheless included as they encode invention polypeptides, arginine-framed tripeptide repeats and fragments thereof.

The invention also includes nucleic acids substantially homologous with the nucleic acids encoding invention polypeptides. As used herein, the term "homologous," when used in reference to nucleic acid molecule, refers to similarity between two nucleotide sequences. When a nucleotide position in both of the DNA molecules is occupied by identical nucleotides, then they are homologous at that

position. Preferably, "substantially homologous" nucleic acid sequences are at least 70% homologous, more preferably at least 80% homologous and most preferably 90% homologous, and retains the biological activity associated with the sequence to which it is homologous. For nucleic acids, the length of comparison between sequences will generally be at least 30 nucleotides, preferably at least 50 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides or more. Algorithms for identifying homologous sequences that account for sequence gaps, mismatches, their length and location, are known in the art, such as BLAST (see *e.g.*, Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990).

The invention nucleic acids are useful for encoding invention polypeptides, when such nucleic acids are incorporated into expression systems disclosed herein or known in the art. In addition, invention nucleic acids are useful as probes which can be used to identify the presence of a nucleic acid related to an invention nucleic acid or to detect the presence or an amount of DNA or mRNA in a sample, for example (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y., 1989). Such probes can be modified so as to be detectable using radionuclides, luminescent moieties etc.

The invention further includes nucleic acid sequences complementary to the sequences described herein, such as antisense. Such single or double stranded RNA sequences (known as "RNA") are useful for inhibiting gene expression, for example (Kennerdell *et al.* (1998) *Cell* 95:1017-1026; Fire *et al.* (1998) *Nature* 391:806-811). Antisense sequences can interfere with a nucleic acid encoding a factor that mediates or contributes to ER retention (*e.g.*, chaperones such as calnexin, hsc70 etc.). An effective amount of an antisense from the coding region of a factor that mediates or contributes to ER retention can be useful for treating physiological disorders associated with export-incompetent proteins including CFTR, as described herein.

Invention nucleic acid sequences can be obtained using various standard techniques known in the art (*e.g.*, molecular cloning, chemical synthesis) and the purity can be determined by polyacrylamide or agarose gel electrophoresis, DNA sequencing and the like. Nucleic acids also can be isolated using hybridization or computer-based techniques, which are well known in the art. Such techniques include, but are not limited to: 1) hybridization of genomic DNA or cDNA libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of

polypeptides expressed by DNA sequences (e.g., using an expression library); 3) polymerase chain reaction (PCR) of genomic DNA or cDNA using primers capable of annealing to a nucleic acid sequence of interest; 4) computer searches of sequence databases for related sequences; and 5) differential screening of a subtracted nucleic acid library.

The nucleic acids of the invention can, if desired, be naked or be in a carrier suitable for passing through a cell membrane (e.g., DNA-liposome complex), contained in a vector (e.g., retroviral vector, adenoviral vectors and the like), or linked to inert beads or other heterologous domains (e.g., antibodies, biotin, streptavidin, lectins, etc.), or other appropriate compositions disclosed herein. Thus, both viral and non-viral means of nucleic acid delivery can be achieved and are contemplated. The nucleic acids of the invention also can contain additional nucleic acid sequences linked thereto that encodes a polypeptide having a distinct functionality, such as the heterologous domains set forth herein. Invention polynucleotides also can be modified, for example, to be resistant to nucleases to enhance their stability in a pharmaceutical formulation, for example.

For propagation or expression in cells, invention nucleic acids can be inserted into a vector. The term "vector" refers to a plasmid, virus or other vehicle known in the art that can be manipulated by insertion or incorporation of a nucleic acid. Such vectors can be used for genetic manipulation (i.e., "cloning vectors") or can be used to transcribe or translate the inserted polynucleotide (i.e., "expression vectors"). A vector generally contains at least an origin of replication for propagation in a cell and a promoter. Control elements, including promoters, present within an expression vector are included to facilitate proper transcription and translation (e.g., splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and, stop codons etc.).

In vivo or *in vitro* expression of the invention nucleic acids can be conferred by a promoter operably linked to the nucleic acid. "Promoter" refers to a minimal nucleic acid sequence sufficient to direct transcription of the nucleic acid to which the promoter is operably linked (see e.g., Bitter *et al.*, Methods in Enzymology 153:516-544, 1987). Promoters can constitutively direct transcription, can be tissue-specific, or can render inducible or repressible transcription; such elements are generally located in the 5' or 3' regions of the native gene.

A "tissue-specific promoter" means a promoter that is active in particular cells or tissues which therefore confers transcription of the operably linked nucleic acid in the particular cells, e.g., liver cells, hematopoietic cells, or cells of a specific tissue within an animal, e.g., pancreatic β cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. An "inducible promoter" means a promoter whose activity level increases in response to treatment with an external signal or agent (e.g., metallothionein IIA promoter, heat shock promoter). A "repressible promoter" or "conditional promoter" means a promoter whose activity level decreases in response to a repressor or an equivalent compound. When the repressor is no longer present, transcription is activated or derepressed. Such promoters may be used in combination and also may include additional DNA sequences that are necessary for transcription and expression, such as introns and enhancer sequences.

As used herein, the term "operably linked" means that a selected nucleic acid (e.g., encoding a substituted export-incompetent CFTR) and a regulatory sequence(s) are connected in such a way as to permit transcription when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s). Typically, a promoter is in close proximity and 5' of the nucleic acid to allow the promoter to regulate expression of the nucleic acid. However, indirect operable linkage is also possible when a promoter on a first plasmid controls expression of a protein that, in turn, regulates a promoter controlling expression of the nucleic acid on a second plasmid.

When cloning in bacterial systems, constitutive promoters such as T7 and the like, as well as inducible promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) may be used. When cloning in mammalian cell systems, constitutive promoters such as SV40, RSV and the like, or inducible promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the mouse mammary tumor virus long terminal repeat, the adenovirus late promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

Mammalian expression systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using

adenovirus expression vectors, the nucleic acid sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used. (see *e.g.*, Mackett et al. (1982) Proc. Natl. Acad. Sci. USA 79:7415-7419; 5 Mackett et al. (1984) J. Virol. 49:857-864; and Panicali et al. (1982) Proc. Natl. Acad. Sci. USA 79:4927-4931).

Mammalian expression systems further include vectors specifically designed for "gene therapy" methods including adenoviral vectors (U.S. Patent Nos. 5,700,470 and 5,731,172), adeno-associated vectors (U.S. Patent No. 5,604,090), herpes simplex 10 virus vectors (U.S. Patent No. 5,501,979) and retroviral vectors (U.S. Patent Nos. 5,624,820, 5,693,508 and 5,674,703 and WIPO publications WO92/05266 and WO92/14829). Vectors based on bovine papilloma virus (BPV) have the ability to replicate as extrachromosomal elements (Sarver et al., (1981) Mol. Cell. Biol. 1:486). Shortly after entry of an extrachromosomal vector into mouse cells, the vector 15 replicates to about 100 to 200 copies per cell. Because transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, a high level of expression occurs. Such vectors also have been employed in gene therapy (U.S. Patent No. 5,719,054). CMV based vectors also are included (U.S. Patent No. 5,561,063).

20 In yeast, a number of vectors containing constitutive or inducible promoters may be used (see *e.g.*, Current Protocols in Molecular Biology, Vol. 2, Ch. 13, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, 1988; Grant et al., "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Vol. 153, pp. 516-544, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., 1987; Glover, DNA Cloning, Vol. II, Ch. 3, IRL Press, Wash., D.C., 1986; Bitter, "Heterologous Gene 25 Expression in Yeast," Methods in Enzymology, Vol. 152, pp. 673-684, Eds. Berger & Kimmel, Acad. Press, N.Y., 1987; and The Molecular Biology of the Yeast Saccharomyces, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II, 1982). A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as 30 GAL may be used ("Cloning in Yeast," R. Rothstein In: DNA Cloning, A Practical Approach, Vol. 11, Ch. 3, Ed. D.M. Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors that facilitate integration of foreign nucleic acid sequences into a yeast chromosome, via homologous recombination for example, are known in the art

and can be used. Yeast artificial chromosomes (YAC) are typically used when the inserted polynucleotides are too large for more conventional yeast expression vectors (*e.g.*, greater than about 12 kb).

Thus, in accordance with the present invention, nucleic acids encoding invention polypeptides may be inserted into an expression vector for expression *in vitro* (*e.g.*, using *in vitro* transcription/translation kits, which are available commercially), or may be inserted into an expression vector that contains a promoter sequence which facilitates transcription in either prokaryotes or eukaryotes by transfer of an appropriate nucleic acid into a suitable cell, organ, tissue or organism.

As used herein, a "transgene" is any piece of nucleic acid that is inserted by artifice into a host cell, and becomes part of the organism that develops from that cell.

A transgene can include one or more promoters and any other DNA, such as introns, necessary for expression of the selected DNA, all operably linked to the selected DNA, and may include an enhancer sequence. A transgene may include a gene that is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Transgenes may integrate into the host cells genome or be maintained as a self-replicating plasmid (*e.g.*, yeast 2 μ plasmids).

As used herein, a "host cell" is a cell into which a nucleic acid is introduced that can be propagated, transcribed, or encoded polypeptide expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication.

Host cells include but are not limited to microorganisms such as bacteria, yeast, insect and mammalian cells. For example, bacteria transformed with recombinant bacteriophage nucleic acid, plasmid nucleic acid or cosmid nucleic acid expression vectors; yeast transformed with recombinant yeast expression vectors; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid); insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus); or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses,

adenovirus, vaccinia virus), or transformed animal cell systems engineered for stable expression.

For long-term expression of invention polypeptides, stable expression is preferred. Thus, using expression vectors that contain viral origins of replication, for example, cells can be transformed with a nucleic acid controlled by appropriate control elements (*e.g.*, promoter/enhancer sequences, transcription terminators, polyadenylation sites, etc.). Although not wishing to be bound or so limited by any particular theory, stable maintenance of expression vectors in mammalian cells is believed to occur by integration of the vector into a chromosome of the host cell. Optionally, the expression vector also can contain a nucleic acid encoding a selectable or identifiable marker conferring resistance to a selective pressure thereby allowing cells having the vector to be identified, grown and expanded. Alternatively, the selectable marker can be on a second vector that is cotransfected into a host cell with a first vector containing an invention polynucleotide.

A number of selection systems may be used, including, but not limited to the herpes simplex virus thymidine kinase gene (Wigler et al. (1977) *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase gene (Szybalska et al., *Proc. Natl. Acad. Sci. USA* 48:2026, 1962), and the adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817, 1980) genes can be employed in tk⁻, hgp⁺ or apr⁺ cells respectively. Additionally, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler et al. (1980) *Proc. Natl. Acad. Sci. USA* 77:3567; O'Hare et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:1527); the *gpt* gene, which confers resistance to mycophenolic acid (Mulligan et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:2072; the *neomycin* gene, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al. (1981) *J. Mol. Biol.* 150:1); and the *hygromycin* gene, which confers resistance to hygromycin (Santerre et al. (1984) *Gene* 30:147). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, In: *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory, ed., 1987).

As used herein, the term "transformation" means a genetic change in a cell following incorporation of DNA (e.g., a transgene) exogenous to the cell. Thus, a "transformed cell" is a cell into which, or a progeny of which, a DNA molecule has been introduced by means of recombinant DNA techniques.

Transformation of a host cell with DNA may be carried out by conventional techniques known to those skilled in the art. For example, when the host cell is a eukaryote, methods of DNA transformation include, for example, calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, and viral vectors.

Eukaryotic cells also can be cotransformed with invention nucleic acid sequences or fragments thereof, and a second DNA molecule encoding a selectable phenotype, as those described herein. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see e.g., Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). When the host is prokaryotic (e.g., *E. coli*), competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method using procedures well known in the art. Transformation of prokaryotes also can be performed by protoplast fusion of the host cell.

In another embodiment, the invention provides non-human transgenic animals having invention nucleic acids. As used herein, the term "animal," when modified by the term "transgenic," refers to an organism that reproduces sexually. Transgenic animals can be produced by methods known in the art.

The term "transgenic animal" refers to any animal whose somatic or germ line cells bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus. The term "transgenic" further includes cells or tissues (i.e., "transgenic cell," "transgenic tissue") obtained from a transgenic animal genetically manipulated as described herein. In the present context, a "transgenic animal" does not encompass animals produced by classical crossbreeding or in vitro fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Invention transgenic animals can be either heterozygous or homozygous with respect to the transgene. Methods for producing transgenic animals are well

known in the art (see for example, U.S. Patent Nos. 5,721,367; 5,695,977; 5,650,298 and 5,614,396).

Preferred transgenic animals contain the transgene integrated into germ cells. Transgenic animals having a transgene integrated into germ cells have the ability to transfer the transgene to offspring. If such offspring in fact possess some or all of the transgene, then they, too, are transgenic animals. Homologous recombination is one mechanism in which a transgene is stably inserted into the genome. Although it is further preferred that the transgene be integrated into the animal's chromosome, the present invention also contemplates the use of extrachromosomally replicating sequences containing a transgene, such as those similar to yeast artificial chromosomes.

In the transgenic animals described herein, the transgene encodes a substituted polypeptide, such as CFTR, for example, or a peptide having an AFT sequence. A particularly useful transgenic would be an animal that exhibits characteristics of cystic fibrosis (*e.g.*, that expresses an export-incompetent CFTR) which has been transformed with a transgene encoding a substituted CFTR or a polypeptide having an AFT sequence. Expression of the transgene can cause animal cells, such as air passageway epithelial cells or pancreatic cells, to increase CFTR expression on the cell surface. Animals that express other export-incompetent polypeptides can be transformed with a polypeptide having an AFT sequence, which can induce or increase intracellular transport of the export-incompetent cell surface polypeptide or secreted polypeptide. Alternatively, transgenic animals transformed with an antisense nucleic acid capable of inhibiting translation of an ER retention factor can exhibit increased export of export-incompetent or other proteins that utilize the intracellular transport pathway. Any animal that can be produced by transgenic technology is included in the invention, although mammals are preferred which include non-human primates, sheep, goats, horses, cattle, pigs, rabbits, and rodents such as mice, guinea pigs, hamsters, rats and gerbils.

Invention polypeptides can be used to generate additional reagents, such as antibodies. Thus, in accordance with the present invention, antibodies that bind to CFTR polypeptides having substitutions as set forth herein, fragments thereof and polypeptides having an AFT sequence are provided. Antibody comprising polyclonal antibodies, pooled monoclonal antibodies with different epitopic specificities, and

distinct monoclonal antibody preparations, are provided. The invention antibodies can be used in diagnostic methods, purification methods and in the treatment methods, as disclosed herein.

The term "antibody" includes intact molecules as well as fragments thereof, such as Fab, F(ab')₂, and Fv which are capable of binding to an epitopic determinant present in an invention polypeptide. Other antibody fragments are included so long as the fragment retains the ability to selectively bind with its antigen.

As used herein, the term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Generally, epitopes have at least five contiguous amino acids.

Antibodies that bind to invention polypeptides can be prepared using intact polypeptide or small peptide fragments thereof as the immunizing antigen. The polypeptide used to immunize an animal is derived from translated DNA or chemically synthesized, if desired, can be conjugated to a carrier protein. Such commonly used carriers that are chemically coupled to the immunizing peptide include, for example, keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid.

Monoclonal antibodies are made by methods well known to those skilled in the art (Kohler *et al.*, *Nature* 256:495, 1975; and Harlow *et al.*, *Antibodies: A Laboratory Manual*, page 726, Cold Spring Harbor Pub., 1988). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by analyzing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques which include, for example, affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see *e.g.*, Coligan *et al.*, "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in: *Current*

Protocols in Immunology sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; and Barnes *et al.*, "Purification of Immunoglobulin G (IgG)," in: Methods in Molecular Biology, Vol. 10, pages 79-104, Humana Press, 1992).

5 The preparation of polyclonal antibodies is well-known to those skilled in the art (see, *e.g.*, Green *et al.*, "Production of Polyclonal Antisera," in: Immunochemical Protocols, pages 1-5, Manson, ed., Humana Press, 1992; Harlow *et al.*, 1988, *supra*; and Coligan *et al.*, 1992, *supra*, section 2.4.1).

10 Antibody fragments (*e.g.*, Fab, F(ab')₂, and Fv) of the present invention can be prepared by proteolytic hydrolysis of the antibody, for example, by pepsin or papain digestion of whole antibodies.

15 In accordance with the present invention, there are provided methods for identifying a therapeutic agent for treating cystic fibrosis. A method of the invention comprises contacting a cell expressing an export-incompetent CFTR with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence, and determining whether the agent increases the amount of export-incompetent CFTR on the cell surface, where an increased amount of CFTR on the cell surface identifies a therapeutic agent for treating cystic fibrosis. Particular export-incompetent CFTRs that may be used include $\Delta F508$ and R1066C.

20 In another embodiment, the invention provides methods for identifying an agent that induces or increases transport of an export-incompetent protein. A method of the invention comprises contacting an export-incompetent protein with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence, and determining whether the agent increases the amount or the rate of protein transported, where an increased amount of the protein transported identifies an agent that induces or increases transport of an export-incompetent protein. In one aspect, the method is performed using a cell expressing an export-incompetent protein. In another aspect, the method is performed in vitro. In certain additional aspects, the export-incompetent protein is a cell surface protein, a secreted protein, a lysosomal protein or an endosomal protein. Such in vitro assays
25
30 are described, for example, in Zhang *et al.* (Nat Struct Biol. 5:180-183 (1998))

As used herein, the terms "transport" or "intracellular transport" describes the movement or progression of a molecule along the ER-golgi pathway. Thus, an induction or increase in "transport" means that the protein is transported from the ER

to the cis-golgi, or beyond, e.g., to the medial-golgi, to the trans-golgi, to the cell surface or, as appropriate, secreted or, to the endosome or lysosome. It is specifically intended that the term transport involve movement from any point within the pathway to any point within the pathway. Preferably, an agent that induces or increases transport will promote movement of the protein to its final destination. For example, an agent that induces or increases CFTR transport will promote movement from the ER to the cell surface. Thus, an "increased rate of transport" or an "increase in the rate of transport" means that the time needed for transport is decreased and an "increase in an amount transported" means that the amount of protein transported is greater.

In the methods of the invention for identifying a therapeutic agent for treating CFTR, or for identifying an agent that induces or increases transport of an export-incompetent protein, an increase in an amount of the protein on the cell surface or an increase in an amount of protein transported will be detected. Various detection methods can be employed. For example, to detect an increase in an amount of protein on the cell surface, immunostaining with a specific antibody (e.g., anti-CFTR, M3A7), or direct visualization (e.g., a CFTR-GFP fusion) as disclosed herein, can be employed. Additional methods useful for determining whether there is an increase in cell surface protein included cell panning. In cell panning assays, plates are coated with an antibody that binds to the cell surface protein. The number of cells that binds to the antibody coated plate corresponds to an amount of protein on the cell surface. To detect an increase in an amount of protein transported, methods that detect an increased amount of protein on the cell surface can be employed. Additionally, as disclosed herein, protein transport can be detected by the acquisition or removal of particular oligosaccharide chains. For example, endoH resistant oligosaccharide chains are acquired in the golgi. Thus, detection of endoH resistant chains provides a means with which to detect transport of protein from the ER to golgi. Later stages of transport (e.g., trans-golgi) are associated with the removal or addition of particular oligosaccharides from proteins. Thus, other glycosidases, such as endoglycosidase F, neuraminidase and the like can be used to detect whether an increase in protein transport to later stage,s such as the trans-golgi, for example, occurs. Such enzymes are commercially available (e.g., Boehringer Mannheim Biochemicals, Indianapolis, IN) or are otherwise known in the art.

Polypeptide sequence motifs that mediate or contribute to ER retention of export-incompetent CFTR are disclosed herein. When cells that express export-incompetent $\Delta F508$ CFTR are treated with polypeptides having a sequence motif such as R-X-R, an increase in CFTR transport from the ER to the cell surface is observed (see for example, Figure 4).

Thus, in accordance with the present invention, there are provided methods for treating cystic fibrosis. A method of the invention comprises administering to a subject a pharmaceutical formulation comprising a polypeptide having an arginine framed peptide sequence in an amount effective for treating cystic fibrosis. In one aspect, the subject expresses an export-incompetent CFTR, such as $\Delta F508$ or R1066C CFTR. In another embodiment, a method of the invention comprises administering to a subject a pharmaceutical formulation containing a CFTR, wherein at least one arginine of the arginine framed peptide sequence is substituted with an amino acid, in an amount effective for treating cystic fibrosis. The substituted CFTR can be wild-type CFTR, mutated CFTR, can be further modified, and can be encoded by a nucleic acid sequence, as disclosed herein. For example, a nucleic acid encoding a CFTR polypeptide having one or more amino acid substitutions of an R-X-R sequence can be administered to a subject afflicted with CF in order to treat CF. Other CFTR mutant sequences are known including, for example, $\Delta I507$, N1303K, S549I, S549R, A559T, H139R, G149R, D192G, R258G, S949L, H949Y, H1054D, G1061R, L1065P, R1066C, R1066H, R1066L, Q1071P, L1077P, H1085R, W1098R, M1101K, M1101R, and can similarly be modified or substituted as set forth herein.

The fact that other proteins destined for the intracellular transport pathway frequently exhibit export incompetency due to mutations, or other factors, indicates that the transport of such export-incompetent polypeptides can be induced or increased by treating with a polypeptide having an AFT sequence. Accordingly, physiological disorders associated with export-incompetent proteins can similarly be treated.

Thus, in accordance with the present invention, there are provided methods for treating a subject having a physiological disorder associated with an export incompetent protein. A method of the invention comprises administering to the subject a pharmaceutical formulation comprising a polypeptide having an R-X-R sequence in an amount effective for treating a physiological disorder associated with

an export-incompetent protein. In one aspect, the polypeptide has at least four amino acids or, in another aspect, at least seven amino acids. In certain other aspects of the invention method, the export incompetent protein is a cell surface protein, a secreted protein, a lysosomal protein or an endosomal protein.

5 Physiological disorders associated with an export incompetent protein that can be treated in a method of the invention include, for example, Stargardt's disease and particular types of macular dystrophy caused by mutations of the retinal rod transporter, ABC-R, resulting in deficiency of ER export. Additional candidate physiological disorders associated with various other ABC (adenine-nucleotide
10 binding cassette) proteins.

 The methods for treating a subject having a physiological disorder associated with an export incompetent protein can be practiced with the invention polypeptides having an AFT sequence based on the CFTR sequence, such as GYRQRLE, EYRYRSV, GQRARIS and QARRRQS. Alternatively, the methods can be practiced
15 using polypeptides having AFT sequence, including flanking sequences, if desired, based upon the amino acid sequence of the export incompetent polypeptide that causes or is associated with the physiological disorder being treated. In this way, increased specificity can be provided by using polypeptides having an AFT polypeptides sequence based on the polypeptide sequence. Increased specificity may
20 decrease potentially deleterious side effects caused by inducing or increasing ER export of unrelated polypeptides, for example, by inhibiting the interaction of an ER retention factor with the unrelated protein thereby increasing its export.

 As the invention polypeptides and nucleic acids are useful for treating physiological disorders associated with an export-incompetent protein, including
25 cystic fibrosis, the present invention also provides pharmaceutical formulations comprising invention polypeptides and nucleic acids.

 The compositions administered to a subject will be in a "pharmaceutically acceptable" or "physiologically acceptable" formulation. As used herein, the terms "pharmaceutically acceptable" and "physiologically acceptable" refer to carriers,
30 diluent, excipients and the like that can be administered to a subject, preferably without excessive adverse side effects (*e.g.*, nausea, headaches, etc.). Such preparations for administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol,

polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobial, anti-oxidants, chelating agents, and inert gases and the like. Various pharmaceutical formulations appropriate for administration to a subject known in the art are applicable in the methods of the invention (e.g., Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA, 1990 and The Merck Index, 12th ed., Merck Publishing Group, Whitehouse, NJ, 1996).

Controlling the duration of action or controlled delivery of an administered composition can be achieved by incorporating the composition into particles or a polymeric substance such as polyesters, polyamine acids, hydrogel, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate, or lactide/glycolide copolymers, polylactide/glycolide copolymers, or ethylenevinylacetate copolymers. The rate of release of the composition may be controlled by altering the concentration or composition of such macromolecules. For example, it is possible to entrap an AFT sequence in micro-capsules prepared by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule complexes, nano-capsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

The compositions administered by a method of the invention can be administered parenterally by injection or by gradual perfusion over time. The composition can be administered via inhalation, intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally, and preferably is administered intravascularly. The compositions can be administered in a single dose, or multiple doses. The doses needed for treating a physiological disorder associated with an export-incompetent protein will be sufficient to ameliorate some or all of the

symptoms of the disorder. Such dosages can readily be determined by those skilled in the art (see for example, Ansel et al. Pharmaceutical Drug Delivery Systems, 5th ed. (Lea and Febiger (1990), Gennaro ed.).

5 The AFT polypeptides increase export incompetent CFTR cell surface expression. Although not wishing to be bound by any particular theory, it is reasonable to assume that the mechanism by which the AFT polypeptide sequence increases export is by competitively inhibiting binding of an ER retention factor to its recognition site. As used herein, the term "ER retention factor" refers to any biological molecule, such as a protein, macromolecule, macromolecular machinery or
10 organelle that mediates or contributes to a protein's retention in the ER, as assessed by an inability or delay in active or passive export or egress of the protein from the ER.

It is possible that such an ER retention factor may also recognize signals other than an AFT. Thus, even though an export-incompetent protein lacks an AFT sequence, polypeptides having an AFT sequence may still competitively inhibit an ER
15 retention factor thereby inducing or increasing transport of a protein that does not have an AFT sequence.

Thus, in another embodiment, the invention provides methods for inducing or increasing intracellular transport of an export-incompetent protein in a cell. A method of the invention comprises contacting a cell with a composition comprising a
20 polypeptide having an R-X-R sequence in an amount sufficient for inducing or enhancing intracellular transport of the export-incompetent protein. In one aspect, the export-incompetent protein has an AFT sequence. In certain other aspects, the export incompetent protein is secreted or is a cell surface protein.

Any cell can be employed in a method of the invention for identifying a CF
25 therapeutic agent or for identifying an agent that induces or increases transport of an export-incompetent protein, so long as the cell expresses or is made to express an export-incompetent protein.

In another embodiment, there are provided methods for identifying an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER
30 retention factor. A method of the invention comprises incubating an R-X-R polypeptide and an ER retention factor under conditions allowing their interaction; adding a test agent to the incubation; and detecting binding between said R-X-R polypeptide and said ER retention factor, where decreased binding in the presence of

the test agent identifies an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER retention factor.

As used herein, the term "incubating" refers to conditions that allow the contact, binding or interaction between a polypeptide, functional fragment or polynucleotides encoding same and the test agent. The term "contacting" as used herein includes in solution, in solid phase, in cells and in an animal.

A therapeutic agent for treating CFTR or other physiological disorders associated with an export-incompetent protein, as well as agents that induce or increase transport of an export-incompetent protein and agents that inhibit, prevent or disrupt the interaction between an R-X-R polypeptide and an ER retention factor can essentially be any molecule. Generally the agent will be a small organic molecule (e.g., a peptide) capable of interacting with either an appropriate polypeptide or ER retention factor such that the agent competitively or non-competitively inhibits, interferes with or antagonizes, partially or completely, the interaction. An example of a competitive antagonist would be an agent that binds to an ER retention factor at the site of interaction with the polypeptide that subsequently prevents interaction with the polypeptide. Alternatively, a competitive antagonist can bind to an ER retention signal present on a polypeptide, such as an AFT, with which the ER retention factor interacts, thereby preventing or inhibiting its interaction with an ER retention factor. An example of non-competitive antagonist would be an agent that binds either an ER retention factor or a polypeptide containing an appropriate retention signal, but does not prevent or inhibit its interaction. Nevertheless, such a non-competitive antagonist prevents or inhibits ER retention of the complex, thereby promoting export.

Although such agents will generally directly interact with the ER retention factor or the polypeptide with which the ER retention factor interacts, agents that function indirectly also are contemplated. For example, an indirect agent may activate or increase an interaction between a polypeptide and a factor that contributes to or mediates ER export, thereby inducing or increasing transport from the ER

Further included are agents that function through any enzymatic interaction in which the agent directly or indirectly performs a biochemical modification (e.g., phosphorylation) of the polypeptide or ER retention factor that results in inhibiting or blocking ER retention. For example, post-translational modification of an amino acid sequence near an AFT sequence may prevent or inhibit its interaction with an ER

retention factor. Thus, agents that directly or indirectly alter polypeptides, such as to increase or decrease phosphorylation, ubiquitination, glycosylation, proteolytic cleavage and the like are therefore included.

AFT polypeptides also can be employed to identify an ER retention factor.

5 Thus, the invention further provides a method for identifying an ER retention factor. A method of the invention comprises contacting a composition suspected of containing an ER retention factor with a polypeptide having an R-X-R sequence under conditions allowing their interaction and detecting binding between the factor and the polypeptide, thereby identifying an ER retention factor. The method can be performed
10 in vitro. The method also can employ polypeptides having various lengths as set forth herein. Specific polypeptides include GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

In various other embodiments, the invention also provides methods for inhibiting degradation of a protein in a cell. A method of the invention comprises
15 contacting a cell with a polypeptide having an R-X-R sequence in an amount sufficient for inhibiting degradation of a cell surface or secreted protein. The invention also provides methods for detecting the presence of an export-incompetent protein in a cell. A method of the invention comprises contacting a cell with a polypeptide having an R-X-R sequence and detecting the intracellular transport of the
20 protein. The various assays for detecting transport have been set forth herein, using an enzymes, such as endoglycosidases, cell surface immunolabeling etc. Such methods are equally applicable in a method for detecting the presence of an export-incompetent protein in a cell.

In accordance with the present invention, there are provided kits for treating
25 subjects having or suspected of having a physiological disorder or condition associated with an export-incompetent protein. A kit of the invention contains one or more AFTs or a polypeptide containing an AFT sequence, and a label or packaging insert for treating a physiological disorder associated with an export-incompetent protein as set forth herein in suitable packaging material. In various aspects, the kits
30 contain R-X-R tripeptides, preferably polypeptides having an R-X-R sequence at least 4 amino acids long, more preferably polypeptides having an R-X-R sequence at least 5 amino acids long, and most preferably polypeptides having R-X-R sequence at least 6 amino acids or longer. It is understood that any invention polypeptide can be included

in the kits of the invention. Particular embodiments include peptides having the sequences GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

As used herein, the term "packaging material" refers to a physical structure housing the components of the kit, such as invention polypeptides and nucleic acids.

5 The packaging material preferably maintains the components sterilely, and can be made of material commonly used for such purposes (e.g., paper, corrugated fiber, glass, plastic, foil etc.). The label or packaging insert indicates that the kit is to be used in a method of the invention, for example, for treating CF.

10 As disclosed herein, the substitution of wild-type CFTR arginine framed peptide sequence or other signaling motifs in the C-terminal extension domain may increase transport of the substituted wild-type CFTR over unsubstituted wild-type CFTR. Thus, an amount of CFTR transported to the cell surface can be increased by such substitutions, even in an export-competent wild-type CFTR. Accordingly, it is contemplated that substituted polypeptides that utilize the intracellular transport
15 pathway can be produced in greater amounts than unsubstituted polypeptides by virtue of this increased transport. For example, secretion of a recombinant polypeptide can be increased by substituting an arginine framed peptide or one of the C-terminal extension motifs in the protein.

20 Thus, in accordance with the present invention, there are provided methods for increasing the biological production of recombinant polypeptides. A method of the invention comprises transforming a host with a nucleic acid encoding a recombinant polypeptide having a substituted arginine framed peptide sequence under conditions allowing production in an amount greater than unsubstituted polypeptide, thereby increasing biological production of the recombinant polypeptide.

25 As used herein, the term "biological production" refers to the production of a polypeptide by a living cell, tissue, organ or entire organism, such as an animal. The methods of the invention for increasing the biological production of useful polypeptides can be applied to naturally occurring cell surface, secreted, endosomal or lysosomal polypeptides. Additionally, the production of polypeptides engineered to
30 utilize the intracellular transport pathway, by attaching an intracellular transport targeting sequence, for example, can similarly be increased.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which

this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims. The invention is further described in the following examples, which do not limit the scope of the invention(s) described in the claims.

EXAMPLE I

This example shows that particular signaling motifs mediate or contribute to the export incompetent characteristic of Δ F508 CFTR.

Inspection of the CFTR amino acid sequence reveals four arginine-framed tripeptide (AFT) sequences, one in the N-terminal cytoplasmic domain, two in the first nucleotide-binding domain (NBD1) and one in the R-domain (Figure 1A). Because of the sensitivity of CFTR to misprocessing due to missense mutations in its cytoplasmic domains (Seibert, F. S et al. (1995) J. Biol. Chem. 270:2158-2162) the most conservative substitutions of these triplets i.e. substitutions of a lysine for the arginine at position 1 or 3, were made (Figure 1B).

Each of the arginine to lysine substitutions were introduced into plasmids containing either the wild-type or Δ F508 CFTR sequences. Briefly, a full-length human CFTR cDNA in pNUT vector (Chang, X.-B. et al. (1993). J. Biol. Chem. 268:11304-11311) was utilized as a template for site-directed mutagenesis (QuickChange Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA) by polymerase chain reaction (PCR) according to the manufacturer's recommendation. The following oligonucleotides were used to introduce R29K, R516K, R555K and R766K into wild-type CFTR cDNA. R29K:

CAATTTTGAGGAAAGGATACAAACAGCGCCTGGAATTGTCAG and
CTGACAATTCCAGGCGCTGTITGTATCCTTTCCTCAAAATTG; R516K:
CCTATGATGAATATAAATACAGAAGCCTCATC and
GATGACGCTTCTGTATTTATATTCATCATAGG; R555K:

GGAGGTCAACGAGCAAAAATTTCTTTAGCAAGAG and
CTCTTGCTAAAGAAATTTTGCTCGTTGACCTCC; R766K:
CTTCAGGCACGAAGGAAGCAGTCTCTCCTGAACC and
GGTTCAGGACAGACTGCTTCCTTCGTGCTGAAG. To combine four lysine
5 substitutions in one cDNA, the following fragments were prepared: Dra III - Kpn I
fragment which contains part of wild-type CFTR cDNA from nt 3328 to 4721 and
most of the pNUT expression vector; Kpn I - Afl II fragment which contains part of
the pNUT expression vector and part of CFTR from nt 72 to 993 covering the R29K
mutation; Afl II - Dra III fragment from nt 994 to 1777 covering the R516K
10 substitution; Dra III - EcoR I fragment from nt 1778 to 2230 covering the R555K
substitution; and EcoR I - Dra III fragment from nt 2231 to 3327 covering the R766K
substitution. These five fragments were ligated together to generate full length CFTR
cDNA in pNUT (T4 DNA ligase, New England Biolabs, Beverly, MA). The
sequences of the four fragments covering these substitutions were verified after
15 insertion into the pNUT-CFTR. To combine the cystic fibrosis causing mutation,
 Δ F508, with each individual or all four of the above substitutions, the same strategy
was utilized, except that pNUT- Δ F508 CFTR was utilized as template. pNUT- Δ F508
CFTR/R29K was made by ligating the following three fragments together, Kpn I - Afl
II fragment which contains part of pNUT vector and part of CFTR from nt 72 to 993
20 covering the R29K substitution, Afl II - Hpa I fragment from nt 994 to 2463 covering
the Δ F508 substitution, and Hpa I - Kpn I fragment which contains the part of CFTR
from nt 2464 to 4721 and part of the pNUT vector. The sequences covering Δ F508,
R29K, R516K, R555K and R766K were verified after insertion into the expression
vector pNUT.

25 Baby hamster kidney cells (BHK) cells grown at 37°C in 5% CO₂ were
employed as hosts for CFTR expression as described previously (Loo et al. (1998)
EMBO J 17:6879-6887; and Seibert, F. S et al. (1995) J Biol Chem 270:2158-2162).
Subconfluent BHK cells were transformed with the above wild-type or Δ F508 CFTR
constructs using calcium phosphate precipitation (Chen, C., and Okayama, H. (1987)
30 Mol Cell Biol 7: 2745-2752). After growth in selective media containing 500 μ M
methotrexate, individual colonies were picked and amplified in the same selective
media.

BHK cells were grown, collected and then lysed with 1% SDS; the DNA was sheared by cycling through a 22 gauge needle. Protein lysate (20 µg) from the sample were then subjected to SDS-PAGE on 7% polyacrylamide gels. Protein was electroblotted from the gel to nitrocellulose membrane and the membrane was probed with a mouse anti-CFTR monoclonal antibody, M3A7 (Kartner, N. et al. (1992) *Nat. Genetics* 1:321-7). Chemiluminescent detection was performed according to the manufacturer's recommendations (Pierce).

For endoglycosidase H treatment, cell lysates were diluted with 10 volumes of buffer (50 mM sodium acetate, pH 5.3, 0.5% Nonidet P-40, 1% β-mercaptoethanol plus a protease inhibitor mixture: 2 µg/ml aprotinin, 120 µg/ml benzamidine, 3.5 µl E64, 1 µg/ml leupepetin and 50 µg/ml pefabloc). This yielded a final SDS concentration of 0.1%. Samples were then incubated for 4 hr at 37°C with 10 mU of endoglycosidase H (Boehringer Mannheim). The reactions were stopped by addition of 4 volumes of cold ethanol (-20°C) to precipitate the proteins. Precipitated proteins were then resuspended in gel loading dye and subjected to SDS-PAGE and immunoblotting as above.

Western blots of BHK cell extracts revealed that these four R→K substitutions individually or in combination did not dramatically change the steady state amounts of mature, fully glycosylated band (~170 kDa) or immature core-glycosylated band (~150 kDa) of wild-type CFTR (Figure 1C, lanes 1-6). Thus, synthesis and processing was not impaired by any of these mutations. In the case of ΔF508 CFTR, however striking changes were observed. Without any additional mutation, it displays the usual doublet band pattern with the same major core-glycosylated band (~150 kDa) as the wild-type plus a smaller band at ~135 kDa (lane 7). The latter is missing an N-terminal fragment of ~15 kDa due to alternative initiation of translation or a specific proteolytic clip and is much more prominent in mutants such as ΔF508 than in wild-type CFTR. This band remains prominent in 3 of the 4 R→K variants (R516K, R555K, R766K; lanes 9, 10, and 11 respectively) but not in R29K (lane 8) or in "4RK (lane 12)." More significantly, however, bands of lower mobility are produced by the R29K and 4RK variants (lanes 8 and 12). These are not identical in size but they appear to be at either edge of the broad band displayed by wild-type CFTR because of heterogeneous N-glycosylation. The relative amount is greater in the 4RK combination mutant.

Endoglycosidase-H insensitivity of these large bands formed by the R29K and 4RK versions of $\Delta F508$ CFTR confirms that indeed their oligosaccharide chains have been extended by Golgi glycosyl transferases (Figure 1D, arrowhead). Further evidence that these bands were present at the cell surface came from their accessibility to an impermeant amino reactive biotinylation reagent added to intact cell monolayers. Therefore, substitution of one of the obligatory arginines, in the RQR triplet is able to partially overcome the maturation arrest of $\Delta F508$; this effect is much more pronounced when similar substitutions are made in all four triplets.

For cell surface biotinylation studies, cells grown to about 90% confluency were treated with 2 mM sodium butyrate for 18 h to augment CFTR expression. Dishes of cells were then placed on ice in a cold room for 30 minutes and all subsequent steps were performed. Cells washed with PBS containing 0.1 mM CaCl_2 and 1.0 mM MgCl_2 , were labeled using 3 ml of 1 mg/ml EZ-Link Sulfo-NHS-SS-biotin (Pierce) in PBS, pH 8.0 for 30 minutes. Following washes in PBS + 1% BSA and PBS, cells were incubated for 30 minutes in NP40 lysis buffer (0.09% NP40, 150 mM NaCl, 10 mM NH_4MoO_4 , 50 mM Tris-HCl, pH 7.4), scraped and transferred to microfuge tubes. Insoluble materials were removed by centrifugation. Biotinylated proteins were then precipitated by incubation overnight with 50 μl of packed immobilized streptavidin beads (Pierce). Beads were washed with RIPA buffer (0.1% SDS, 1% DOC, 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and proteins eluted with gel sample buffer for electrophoresis and Western blotting with M3A7 as detailed above.

The results in Figure 1D show that the mature band of substituted $\Delta F508$ CFTR ($\Delta F508/4RK$) was accessible to the impermeant amino reactive biotinylation reagent added to the intact cell monolayers. These results confirm that substitution of AFTs can overcome the biosynthetic arrest of $\Delta F508$ CFTR

EXAMPLE II

This example shows that export of substituted wild-type and $\Delta F508$ CFTR from the endoplasmic reticulum and subsequent maturation is kinetically increased in comparison to unsubstituted wild-type and $\Delta F508$ CFTR.

Pulse chase studies were performed to determine the rate at which maturation occurred in the arginine-framed triplet substituted wild-type and $\Delta F508$ CFTR

polypeptides (Figure 2). Briefly, cells expressing unsubstituted and substituted wild type or $\Delta F508$ CFTR were starved for 30 minutes by incubation in methionine-free medium and then labeled for 20 minutes with 100 $\mu\text{Ci/ml}$ of ^{35}S -methionine (> 800 Ci/mmol; Amersham). Cells were lysed at the end of the labeling period (0 chase time) or were further incubated in complete medium containing 5% fetal bovine serum and 1 mM methionine for the indicated times (chase). Cells were collected and lysed with the NP40 lysis buffer described above and centrifuged at $15,000 \times g$ for 15 min at 4°C to obtain a soluble supernatant which was incubated overnight with the M3A7 primary antibody. Protein G-agarose (Gibco-BRL) was then used to remove the complexes formed which were washed four times with RIPA buffer, dissolved in electrophoresis sample buffer, and subsequently fractionated on SDS-7% acrylamide gels. Following fractionation, the gels were fixed in 30% methanol and 10% acetic acid, equilibrated in 1M sodium salicylate and dried for fluorography. Electronic autoradiography was performed with a Packard Instant Imager. Conversion of ^{35}S -methionine pulse labeled precursor form of CFTR (time 0) to mature product after chase times (1, 2, 3 and 5 hour) are indicated.

At the end of the 30 min pulse (0 min), maturation of the wild-type CFTR precursor substituted with four lysine substitutions (wild-type/4RK) was already evident. In contrast, no maturation for unsubstituted wild-type CFTR was observed at the end of the pulse (compare Figure 2A to 2B).

Conversion of the substituted $\Delta F508/4\text{RK}$ CFTR precursor to mature endoH resistant oligosaccharide chains is readily apparent whereas with $\Delta F508$ CFTR, there is no conversion of the core-glycosylated precursor to any higher molecular weight product (compare Figure 2C to 2D). Electronic autoradiography of the same dried gels indicated that approximately 10% of the radioactivity in the pulse-labeled immature $\Delta F508/4\text{RK}$ band (0 time) was present in the larger mature band after 4 h of chase (Figure 2D). This compares with $\sim 30\%$ conversion of precursor to product in the case of wild-type CFTR. Hence, the extent of maturation of $\Delta F508/4\text{RK}$ viewed in this way is generally similar to the steady-state proportion reflected in the immunoblots (Figure 1C). Furthermore, substitution of all four AFTs markedly decreased degradation and promoted maturation of $\Delta F508$.

These results indicate that the rate of ER export of export-competent and export-incompetent polypeptides such as CFTR can be increased by substitution of AFT sequences as set forth herein.

EXAMPLE III

This example shows that increased maturation of substituted wild-type and Δ F508 CFTR can be observed directly in cells.

Immunofluorescence microscopy in cells expressing Δ F508 CFTR was performed using the highly specific monoclonal antibody, M3A7 (Kartner et al., 1992, supra). Briefly, transformed cells were grown on coverslips, fixed in 70% cold methanol at -20°C for 10 minutes, blocked using 1% BSA and 5% normal rabbit serum in PBS and were permeabilized by incubating in PBS + 0.1% saponin for 1 h at 4°C . CFTR was detected by incubation with 10 $\mu\text{g/ml}$ of M3A7 primary antibody for 60 min at room temperature. Rhodamine linked rabbit anti-mouse secondary antibody (DAKO) was diluted 1:50 in the same blocking solution for a further 1 h incubation. Photomicrographs were made using a Diagnostic Instruments Spot Cam digital camera on a Nikon Microphot-FXA microscope.

The results in Figure 3 indicate that four R \rightarrow K substitutions of wild-type CFTR does not alter its normal distribution, with perinuclear staining indicating ER localization of the immature CFTR in addition to strong uniform staining of mature CFTR over the entire cell surface (Figure 3A and 3B). Perinuclear staining only was observed in cells expressing Δ F508 CFTR indicating ER retention (Figure 3C). In contrast, there is extension of the staining more peripherally in cells expressing Δ F508/4RK CFTR (Figure 3D). Although there is still less peripheral staining for Δ F508/4RK CFTR than for wild-type CFTR, this is expected based upon the relative amounts of the mature CFTR with complex oligosaccharide chains (Figure 1C and Figure 2).

EXAMPLE IV

This example shows that polypeptides containing an AFT sequence can induce or enhance transport of export incompetent Δ F508 CFTR from the endoplasmic reticulum to the cell surface.

Heptamer peptides including two residues from the CFTR sequence on either side of the AFT were synthesized (Molecular Biology Core of the Mayo Clinic, Rochester, MN). BHK cells expressing either wild-type or $\Delta F508$ CFTR-GFP fusion protein (Loo et al., 1998, supra) were allowed to take up a mixture of the heptamers by scrape loading (Malcolm et al., (1996) L Biol Chem, 271:13135-9) and plated on cover slips for visualization the next day using a fluorescence microscope with an FITC filter. Localization of wild-type CFTR-GFP fusion with or without exposure to heptamers was the same as antibody staining of wild-type CFTR. Cells expressing the $\Delta F508$ CFTR-GFP fusion protein in the absence of heptamers also exhibited the same perinuclear clustering as was observed with antibody staining of unfused $\Delta F508$ CFTR. In contrast, heptamer treatment of cells expressing the $\Delta F508$ CFTR-GFP fusion protein exhibited more peripheral fluorescence staining.

The results in Figure 4 show that an AFT sequence can competitively inhibit recognition of AFT containing nascent polypeptides thereby inhibiting ER retention and promoting transport of polypeptides containing AFT sequence from the endoplasmic reticulum.

EXAMPLE V

This example shows that the R \rightarrow K substitutions result in a functionally active $\Delta F508$ CFTR at the cell surface.

Wild-type and $\Delta F508$ CFTR expressing cells with each of the individual R \rightarrow K substitutions, or all four lysine substitutions were grown to confluence in six well culture dishes. The cells were washed with a modified Ringer's buffer solution (136 mM NaNO₃, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 2 mM Mg(NO₃)₂, 10 mM glucose and 20 mM HEPES, pH 7.4) and loaded for 60 min at room temperature with the same buffer supplemented with 1 μ Ci Na³⁶Cl (Amersham) in a volume of 0.5 ml per well. Wells were then washed three times at one min intervals. Samples (0.5 ml) were then collected at 1 minute intervals for scintillation counting with stimulation occurring at time 0 by adding buffer containing 1 mM IBMX, 10 μ M Forskolin and 100 μ M dibutyryl cyclic AMP. The 0.5 ml samples were collected into 24 well Top Count plates (Packard) and 1 ml of Microcint 40 scintillation cocktail (Packard) was added. ³⁶Cl radioactivity was determined in a Packard Top Count scintillation counter.

The influence of each of the R→K substitutions on the cAMP stimulated efflux of $^{36}\text{Cl}^-$ from wild-type or ΔF508 CFTR expressing cells loaded with $^{36}\text{Cl}^-$ anion is shown in Figure 5A. Within 3 minutes of cAMP stimulation there was a maximal rate of efflux from all cells expressing wild-type CFTR with or without any or all of the R→K mutants. No detectable increase in efflux rate from cells expressing ΔF508 CFTR alone or with the R516K, R555K or R766K substitution was observed. In cells expressing $\Delta\text{F508}/\text{R29K}$, low rates of $^{36}\text{Cl}^-$ efflux occurred at delayed times after stimulation. In cells expressing ΔF508 CFTR combined with all four R→K substitutions (4RK) the maximal rates of efflux observed were more than half as rapid as those from cells expressing wild-type CFTR, confirming that mature $\Delta\text{F508}/4\text{RK}$ CFTR does function at the cell surface.

To further characterize the Cl^- channel activity, membrane vesicles were isolated from cells expressing wild-type, wild-type/4RK and $\Delta\text{F508}/4\text{RK}$ CFTR, fused with planar lipid bilayers and single channels were recorded (Aleksandrov, A. A., and Riordan, J. R. (1998) FEBS Lett. 431:97-101). Vesicles were prepared and phosphorylated with PKA as described previously (Aleksandrov and Riordan, 1998, supra). Briefly, planar lipid bilayers were painted onto a 0.2 mm hole drilled in a Teflon cup using a phospholipid solution in n-decane containing a 2:1 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (20 mg/ml). The lipid bilayer separated 1.0 ml of solution in the Teflon cup (*cis*-side) from 4.0 ml of solution in an outer glass chamber (*trans*-side). Both chambers were magnetically stirred and thermostated. Heating and temperature control were established using a Temperature Control System TC2BIP (Cell MicroControls). Electrical contact with the solutions was provided by Ag/AgCl electrodes through agar bridges filled with 0.5 M KCl. The membrane potential difference was measured as the difference between *trans* and *cis* side potentials. The *trans* side was grounded and electrical measurements of the single channel current were performed under voltage clamp conditions using an Axopatch 200B (Axon Instruments) amplifier. The output signal was filtered with an 8-pole low pass Bessel Filter LPBF-48DG (NPI Electronic GmbH) with cut off frequency of 50 Hz and recorded on magnetic tape using a VR-10B (Instrutech Corp.) digital data recorded on a Sony SLV-440 VCR. For data analysis, the signal was digitized (Digidata 1200; Axon Instruments) with a sampling rate of 500 Hz and analyzed using pCLAMP 6.0

(Axon Instruments) software. Origin 4.0 (Microcal) software was used to fit all points histograms by multi-peak Gaussians. Membrane vesicles were prephosphorylated with PKA and added as a concentrated stock solution to the *cis* side to obtain a final protein concentration of 10-15 $\mu\text{g/ml}$. Measurements were made in symmetrical salts solutions containing, in mM: 300 Tris-HCl, 2 MgCl, 1 EGTA, pH = 7.2, at a membrane potential of -75 mV. MgATP (2 mM) was added to the *cis* side only. Under these conditions any cationic channels present in the membrane vesicles are invisible and CFTR chloride channels appeared only after fusion of vesicles with an inside out orientation. Conductances for wild-type, wild-type/4RK and $\Delta\text{F508/4RK}$ were 10.7, 10.8, and 10.7 pS, respectively. Mean open times τ_0 were 230, 1400, and 700 msec respectively. Short mean closed times τ_{c1} were 10 msec in each case. Long mean closed times τ_{c2} were 680, 2000, and 2800 msec, respectively. Conductance as a function of Cl^- concentration and temperature as well as anion selectivity of the CFTR ion channel were tested in control experiments showing good agreement with published data (Tabcharani, J. A et al. (1997) *J. Gen. Physiol.* 110:341-54).

Compared with wild-type single channels which exhibit an open probability of ~ 0.25 at 25°C , channels generated by wild-type/4RK had a higher mean open probability of ~ 0.61 (Figure 5B). Thus although the gating kinetics are somewhat altered with both mean open and mean closed times increased, the four R \rightarrow K substitutions certainly do not impede current flow through the channel, consistent with the undiminished rate of ion flux (Figure 5A). Significantly, the $\Delta\text{F508/4RK}$ channels had an open probability similar to wild-type CFTR, confirming the activity revealed by the macroscopic flux measurements. No tracing is shown for ΔF508 CFTR alone as we have never been able to detect CFTR-like single channels using vesicles from BHK cells expressing this variant. We conclude that the channels observed from $\Delta\text{F508/4RK}$ CFTR are entirely due to the maturation that is enabled by the release from ER retention when the AFTs are substituted.

The above examples show that substitution of one of the arginines in each of the four AFTs, individually or simultaneously, permits nascent ΔF508 CFTR to mature about one-third as efficiently as wild-type CFTR and generates functional chloride channels at the cell surface. Thus, release of ΔF508 CFTR from ER retention by interfering with recognition of these tripeptide signals may provide the basis of a novel therapeutic strategy for cystic fibrosis.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

5

What is claimed is:

1. An isolated polypeptide comprising a polypeptide having the sequence X_1 -R- X_2 -R- X_3 , wherein X_1 , X_2 and X_3 are any amino acid.
2. The polypeptide of claim 1, wherein said polypeptide has at least seven amino acids.
3. An isolated polypeptide selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.
4. The polypeptide of claims 1, 2 or 3, further comprising a pharmaceutical formulation.
5. An isolated polypeptide having one or more R-X-R sequences, wherein at least one R of an R-X-R sequence has been substituted with another amino acid, and wherein the substituted polypeptide is exported from the ER in an amount or at a rate greater than the unsubstituted polypeptide.
6. The isolated polypeptide of claim 5, wherein said polypeptide is CFTR.
7. A polynucleotide encoding the polypeptide of claims 1, 2 or 3.
8. A method for identifying a therapeutic agent for treating cystic fibrosis comprising:
 - (a) contacting at least one cell expressing an export-incompetent CFTR with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence; and
 - (b) determining whether the agent increases the amount of said CFTR on the cell surface, where an increased amount of CFTR on the cell surface identifies a therapeutic agent for treating cystic fibrosis.
9. A method for identifying an agent that induces or increases transport of an export-incompetent protein comprising:
 - (a) contacting an export-incompetent protein with a test agent under conditions allowing an interaction between the agent and a factor mediating or contributing to export-incompetence; and
 - (b) determining whether the agent increases the amount or the rate of protein transported, where an increased amount of transported protein identifies an agent that induces or increases transport of an export-incompetent protein.
10. The method of claim 9, wherein said protein is a cell surface protein.
11. The method of claim 10, wherein said protein is CFTR.
12. The method of claim 9, wherein said protein is a secreted protein.
13. The method of claim 9, wherein said export-incompetent protein is expressed in a cell.

14. The method of claim 9, wherein said method is performed in vitro.
15. A method for treating a subject having cystic fibrosis, comprising administering to the subject a pharmaceutical formulation comprising a polypeptide having an R-X-R sequence in an amount effective for treating cystic fibrosis.
- 5 16. The method of claim 15, wherein the X amino acid of the R-X-R sequence is not alanine, asparagine or glutamate.
17. The method of claim 15, wherein said polypeptide has at least four amino acids.
- 10 18. The method of claim 15, wherein said polypeptide has at least seven amino acids.
19. The method of claim 15, wherein said polypeptide is selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.
- 15 20. A method for treating a subject having cystic fibrosis, comprising administering to the subject a pharmaceutical formulation comprising a nucleic acid encoding a polypeptide having an R-X-R sequence in an amount effective for treating cystic fibrosis.
- 20 21. A method for treating a subject having or suspected of having a physiological disorder associated with an export-incompetent protein, comprising administering to the subject a pharmaceutical formulation comprising a polypeptide having an R-X-R sequence in an amount effective for treating a physiological disorder associated with an export-incompetent protein.
- 25 22. The method of claim 21, wherein said physiological disorder or condition is selected from the group consisting of: macular dystrophy and Stargardt's disease.
23. The method of claim 21, wherein said export-incompetent protein is selected from the group consisting of: ion channels, ABC proteins, growth factors, immune regulators, adhesion proteins, hormones, clotting factors, hemostatic regulators and receptors thereof.
- 30 24. The method of claim 21, wherein the X amino acid of the R-X-R sequence is not alanine, asparagine or glutamate.
25. The method of claim 21, wherein said polypeptide has at least four amino acids.
26. The method of claim 21, wherein said polypeptide has at least seven amino acids.
- 35 27. The method of claim 21, wherein said polypeptide is selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.
28. A method for inducing or increasing intracellular transport of an export-incompetent protein, comprising contacting a cell expressing an export-incompetent protein with a composition comprising a polypeptide having an R-X-R

sequence in an amount sufficient for inducing or enhancing intracellular transport of the export-incompetent protein.

29. The method of claim 28, wherein said protein has an R-X-R sequence.
30. The method of claim 28, wherein said protein is a cell surface protein.
- 5 31. The method of claim 30, wherein said protein is an export-incompetent CFTR.
32. The method of claim 28, wherein said protein is secreted.
33. A method for identifying an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER retention factor comprising:
- 10 (a) incubating a polypeptide having an R-X-R sequence and an ER retention factor under conditions allowing their interaction;
- (b) adding a test agent to said incubation; and
- (c) detecting binding between said polypeptide and said ER retention factor, where decreased binding in the presence of the test agent identifies an agent that inhibits or disrupts an interaction between an R-X-R polypeptide and an ER retention factor.
- 15 34. The method of claim 33, wherein said method is performed in vitro.
35. The method of claim 33, wherein said polypeptide has at least 4 amino acids.
- 20 36. The method of claim 33, wherein the X amino acid of the R-X-R sequence is not alanine, asparagine or glutamate.
37. The method of claim 33, wherein said polypeptide is selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.
38. A method for identifying an ER retention factor comprising:
- 25 (a) contacting a composition suspected of containing an ER retention factor with a polypeptide having an R-X-R sequence under conditions allowing interaction between said factor and said polypeptide; and
- (b) detecting binding between said factor and said polypeptide, thereby identifying an ER retention factor.
- 30 39. The method of claim 38, wherein said method is performed in vitro.
40. The method of claim 38, wherein said polypeptide has at least four amino acids.
41. The method of claim 38, wherein said polypeptide has at least seven amino acids.

42. The method of claim 38, wherein said polypeptide is selected from the group consisting of: GYRQRLE, EYRYRSV, GQRARIS and QARRRQS.

43. A method for inhibiting degradation of a protein in a cell comprising contacting a cell with a polypeptide having an R-X-R sequence in an amount sufficient for inhibiting degradation of a cell surface or secreted protein.

44. The method of claim 43, wherein said protein is a cell surface protein.

44. The method of claim 43, wherein said protein is secreted.

45. A method for detecting the presence of an export-incompetent protein in a cell comprising contacting a cell with a polypeptide having an R-X-R sequence and detecting the intracellular transport of the protein

46. The method of claim 45 wherein said intracellular transport is detected using an enzyme.

47. The method of claim 45 wherein said intracellular transport is detected by detecting the presence of the protein on the cell surface.

48. The method of claim 45 wherein said intracellular transport is detected by detecting secretion of the protein.

49. The method of claim 45, wherein said protein is CFTR.

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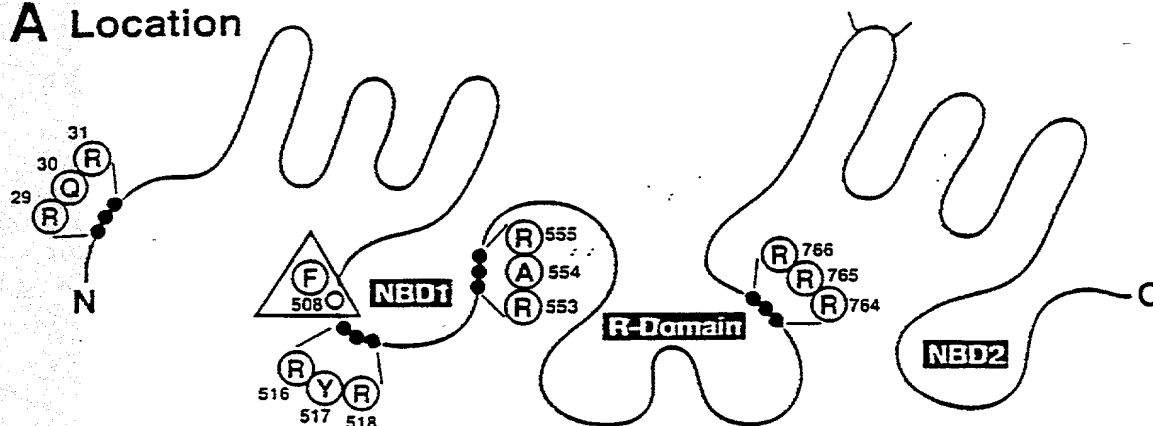
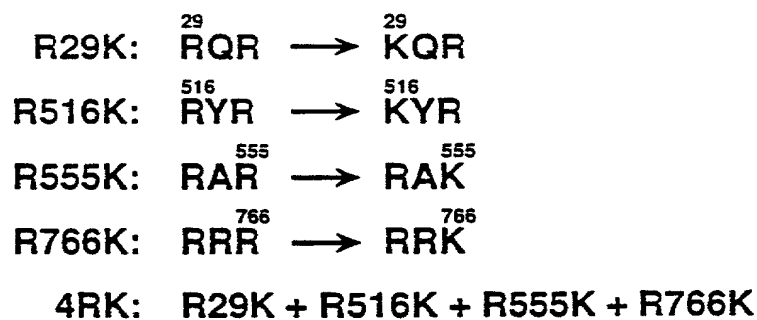
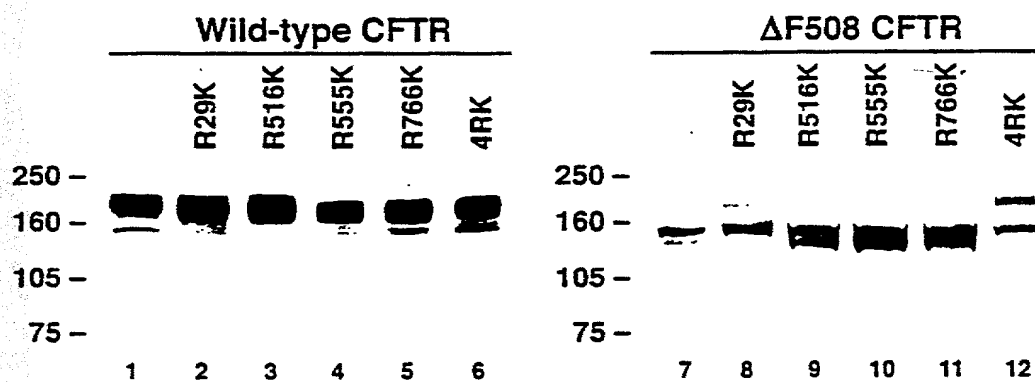
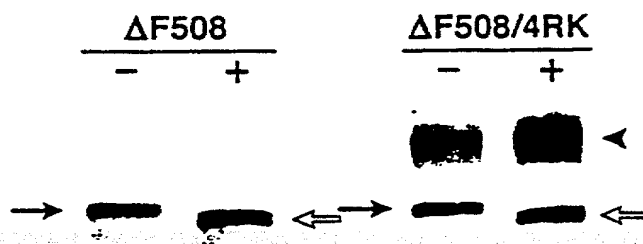
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(54) Title: **CFTR POLYPEPTIDES, FRAGMENTS THEREOF AND METHODS OF USE TO OVERCOME BIOSYNTHETIC
MISPROCESSING**

(57) Abstract: The invention relates to biosynthetic maturation of cell surface polypeptides and, more specifically, to particular CFTR polypeptides which exhibit increased transport to the cell surface and tripeptide amino acid sequences that promote or enhance transport of export-incompetent CFTR to the cell surface.

WO 01/03722 A1

A Location**B Mutagenesis****C Western Blot Analysis****D Endo-H Digestion****FIGURE 1**

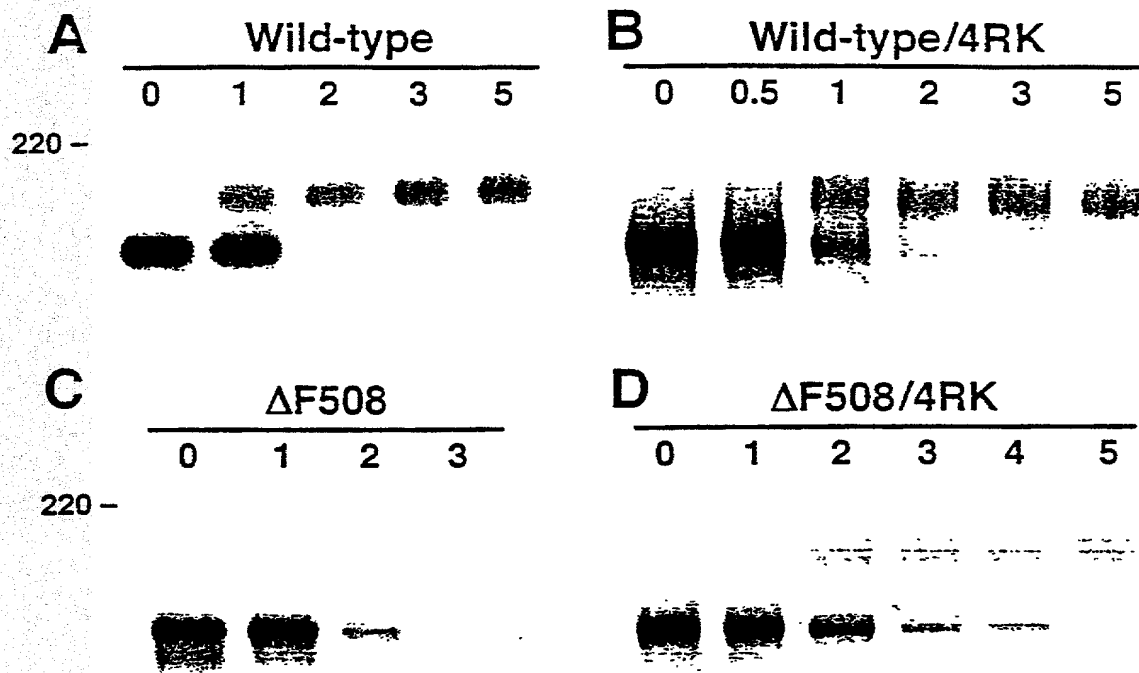
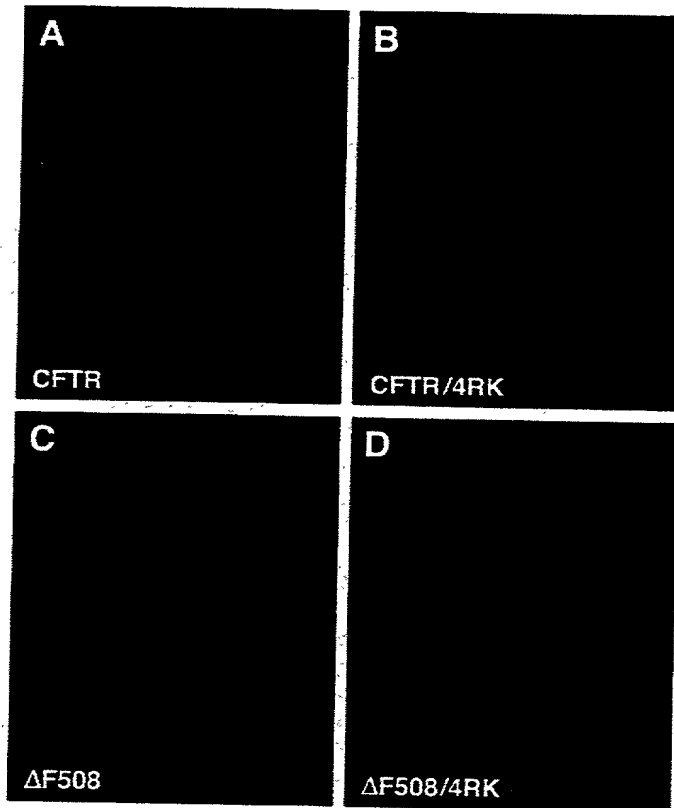
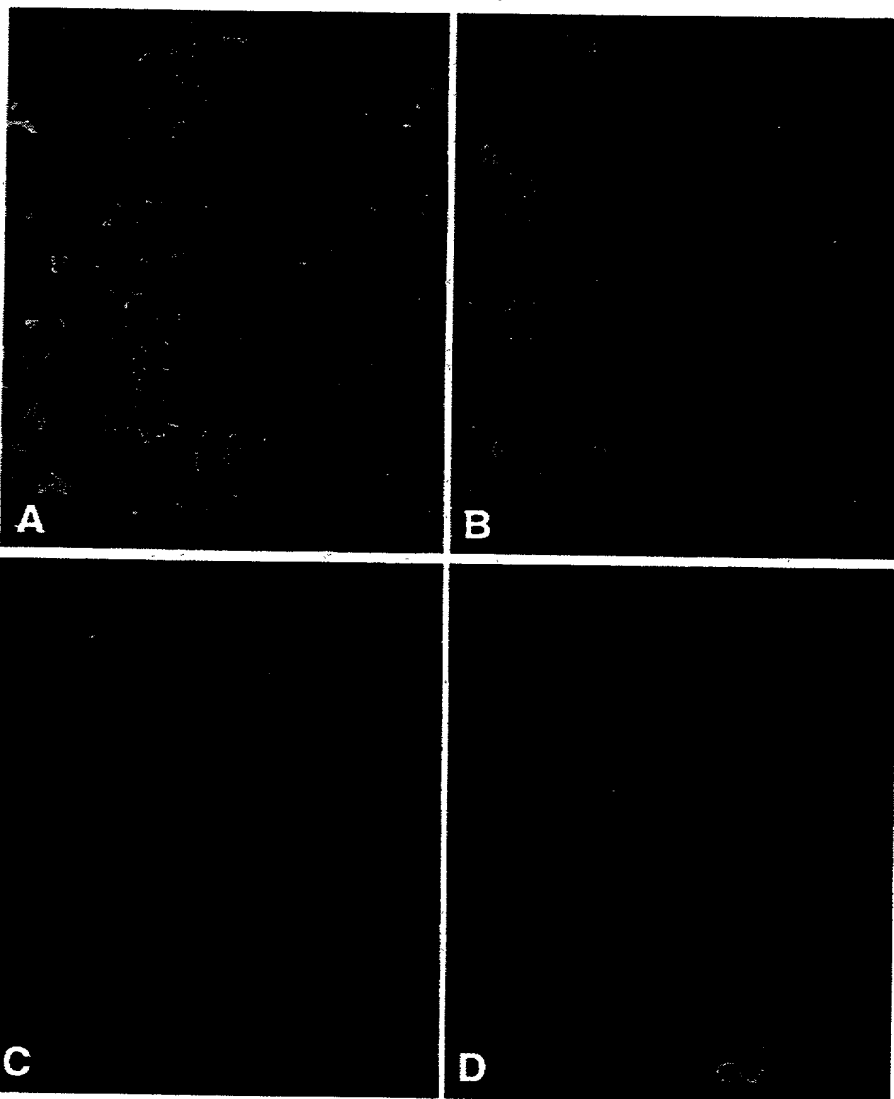


FIGURE 2





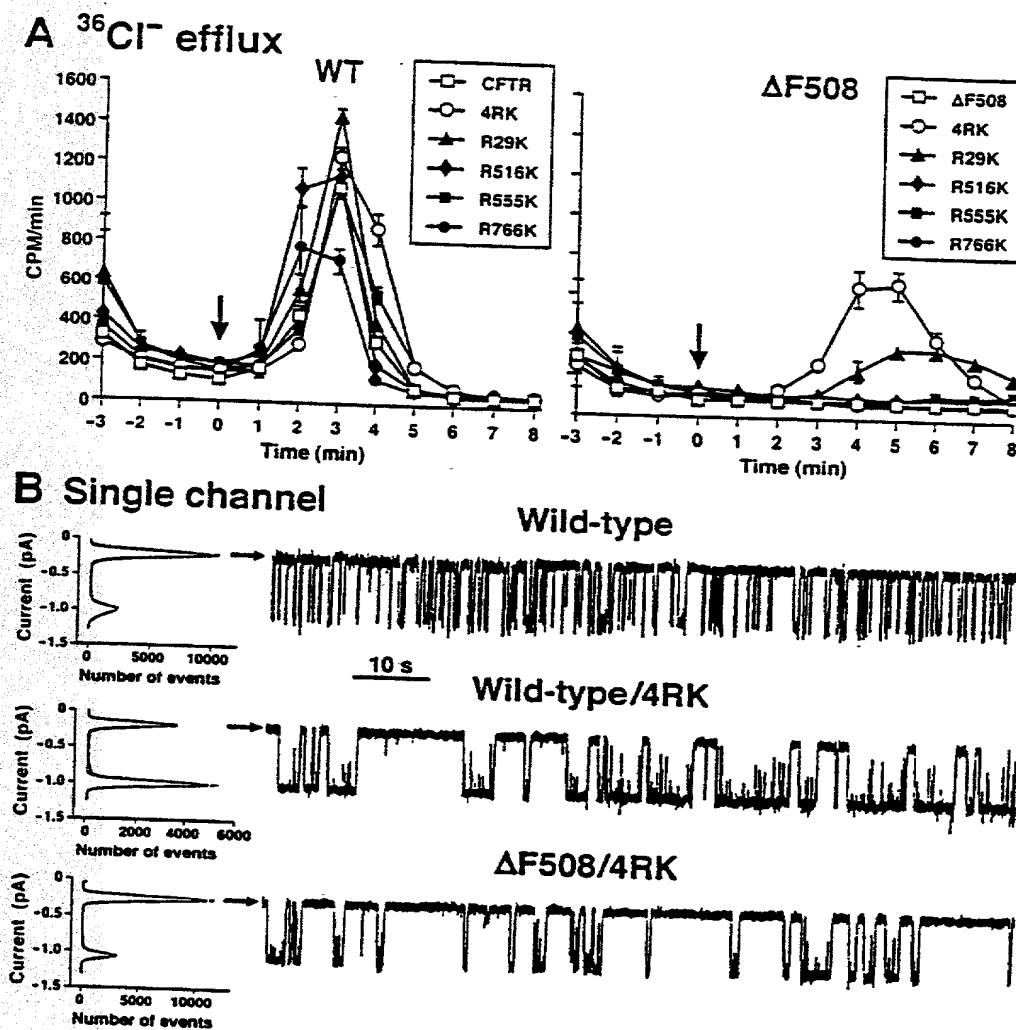


FIGURE 5

HUMAN CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

1 mqrsplekas vvsklffswt rpilrkgyrq rleisdiyqi psvdsadnls eklerewdre
 61 laskknkpli nalrrcffwr fmfygifyl gevtkavqpl llgriasyd pdnkeersia
 121 iylgiglcil fivrtllhp aifglhhigm qmriamfsli ykktklssr vldkisigql
 181 vsllsnlnk fdeglalahf vwiaqlqval lmgliewellq asafcglgfl ivlalfqagl
 241 grmmmkkyrdq ragkiserlv itsemieniq svkaycweea mekmienlrq telkltrkaa
 301 yvryfnssaf ffsghfvvfl svlpyalikg iilrkifti sfcivlmav trqfpwavqt
 361 wydisgank iqdfllqkqey ktleynttt evvmenvtaf weegfgelfe kakqnnnrk
 421 tsngddslff snfslgtpv lkdnfkier gqllavagst gagktsilmm imgelepseg
 481 kikhsgrisf csqfswimpg tikeniihgv sydeyryrsv ikacqleedi skfaekdniv
 541 lgeggitlsg qqrarislar avykdadlyl ldspfgyl dv ltekeifesc vcklmanktr
 601 ilvtskmehl kkadkililh egssyfygtf selqnlqpdf ssklmgcdsf dqfsaerns
 661 iltetlhrfs legdapvswt etkkqsfkqt gefgekrkns ilnpinsirk fsivqktpq
 721 mngieedsde plerrslvp dseqgeailp risvistgpt lqarrqsvl nlmthsvnqg
 781 qnhrkttas trkvsalapqa nlteldiysr rlsqetglei seeineedlk ecffddmesi
 841 pavttwntyl rytvvhksli fvliwclvif laevaaslvv lwlgnstplq dkgnthsrn
 901 nsyaviitst ssyyvfyiyv gvadtllamg ffrgipivht litvskilhh kmhsvlqap
 961 mstlntlkag glnrfskdi ailddllplt ifdfiqllli vigaiavvav lqpyifvatv
 1021 pvivafimlr ayflqtsqql kqlesegrsp ifthlvtslk glwtlrafr qpyfetlthk
 1081 alnlhtanwf lystlrwfq mriemifvif fiavtfisil ttgegegrvg iiltlamnim
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 1201 ddiwpsggqm tvkdltakyt eggnaileni sfsispqgrv gllgrtgsgk stilsafirl
 1261 integeiqid gvswdsitlq qwrkafgvip qkvfifsgtf rkndpyeqw sdqeiwkvad
 1321 evglrsvieq fpgkldfviv dggevlshgh kqlmclarsv lskakilld epsahldpvt
 1381 yqiirrtlkq afadctvilc ehrieamlec qqflvienk vrqydsiqkl lnerslfrqa
 1441 ispsdrvklf phrnsskcks kpqiaalkee teeevqdtrl

FIGURE 6

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled CFTR POLYPEPTIDES, FRAGMENTS THEREOF AND METHODS OF USE TO OVERCOME BIOSYNTHETIC MISPROCESSING, the specification of which:

☐ is attached hereto.

☒ was filed on January 8, 2002 as Application Serial No. 10/030,585 and was amended on _____.

☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119(e)(1) of any United States provisional application(s) listed below:

<u>U.S. Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
60/143,090	July 9, 1999	Abandoned
60/146,097	July 21, 1999	Abandoned

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

<u>U.S. Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
PCT/US00/40324	July 7, 2000	National Phase

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>	<u>Priority Claimed</u>
none			

Combined Declaration and Power of Attorney
Page 2 of 2 Pages

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

19
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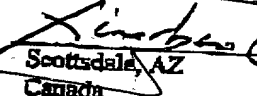
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

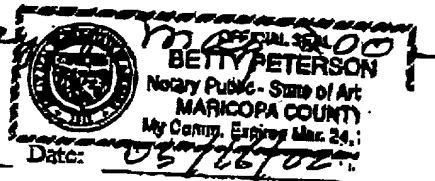
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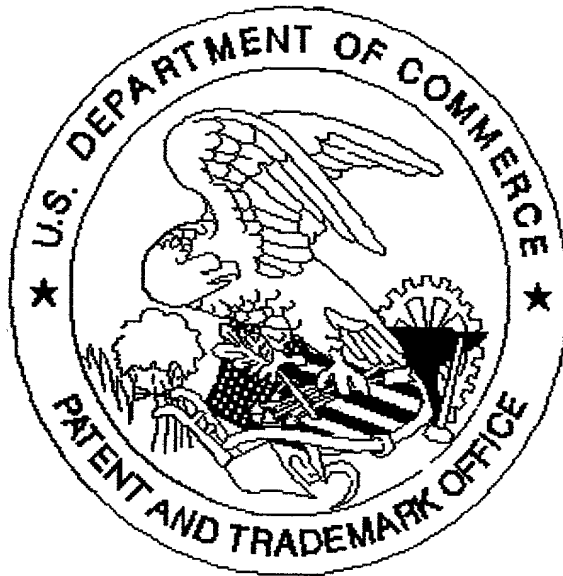
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